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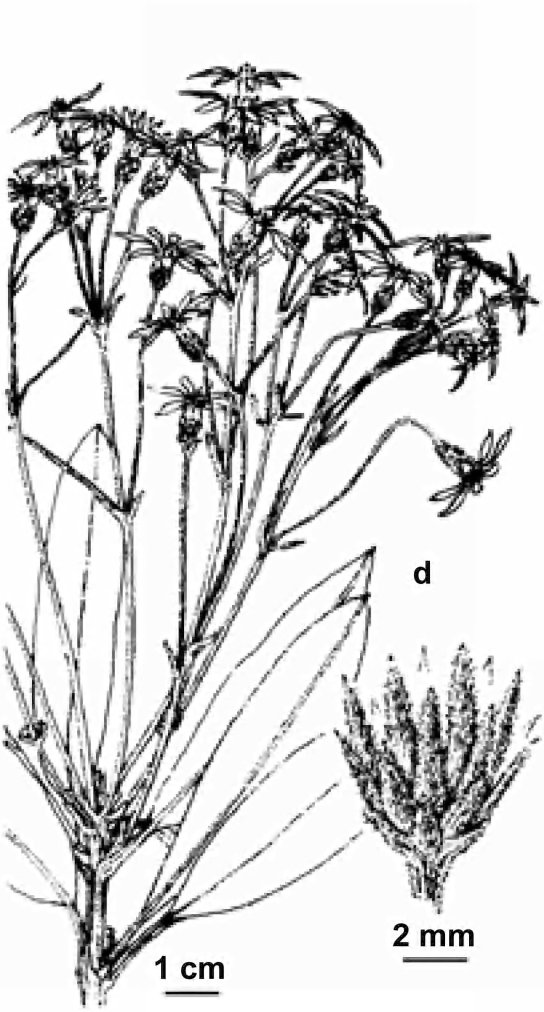

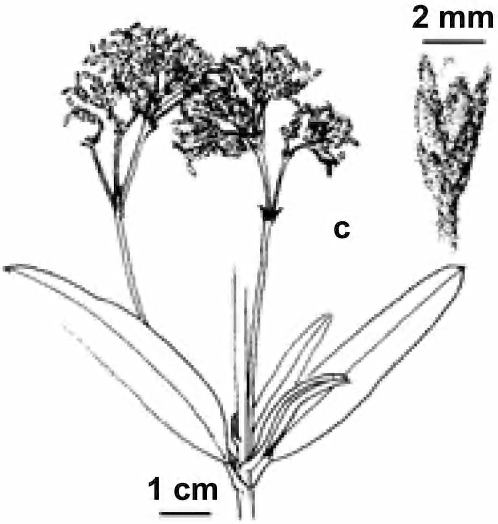

Left: ***Olearia megalophylla*** **(F.Muell.) F.Muell. ex Benth.,** ***Fl. Austral.*** **3: 467 (1867). Right:** ***O. aglossa*** **(Maiden & Betche) Lander,** ***Telopea*** **4(2): 146 (1991).**
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ROYAL BOTANIC
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Challenges to developing a reference sequence database using mass parallel sequencing and lichen herbarium specimens: a case study for microlichens of the Australian Capital Territory

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Abstract

Molecular species identification is a powerful tool in taxonomy, biodiversity research and ecology, in particular for groups of organisms with limited diagnostic features. This tool relies on the development of high-quality reference sequence databases, and such databases can be built using collection specimens and mass parallel sequencing. Here, lichen herbarium specimens and mass parallel sequencing were used to generate reference ITS sequences for microlichens from the Australian Capital Territory (ACT) and to develop a reference sequence database. The preliminary database was then tested on un-identified specimens collected during the 2018 ACT BushBlitz expedition. Challenges met during both database development and molecular species identification suggest that these processes will not be straightforward for microlichens, due to the high number of sequences generated for non-target species (lichen-associated fungi, co-occurring lichenised species and sample cross-contaminants).

***Keywords*:** Australia, ITS barcode, PacBio amplicon sequencing, collection specimens

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Introduction

Lichens, symbiotic associations between a fungus and a microalga (either a green alga or a cyanobacterium, or both), come in all sizes and shapes. Their vegetative structure, called a thallus, ranges from a few millimetres to tens of centimetres in extent. Microlichens, or lichens with small and/or thin thalli, mostly include crustose, squamulose or granulose species, whereas macrolichens, or lichens with large and often bushy thalli, mostly include foliose and fruticose species. Such as for macrolichens, several factors make microlichens difficult to identify to the species level using morphological characters. Diagnostic features are sparse and may be lacking in immature specimens, or misleading in old specimens or specimens growing in atypical conditions. Even when present, critical diagnostic features, such as the ascus tip and ascospore structure, are often difficult to observe and may require the preparation of numerous cross-sections for microscopic examinations. Additionally, morphological convergence and high infraspecific morphological variability are common in lichens, both for vegetative and reproductive structures, making morphological species delimitation and, as a result, morphological identifications, rather difficult, in particular when conducted in the field. Due to their small sizes, all these issues are compounded for microlichens, and their taxonomy lags behind compared to the taxonomy of macrolichens.

In the Australian Capital Territory (ACT), lichens have historically been well studied thanks to the work of local lichen taxonomists (e.g. McCarthy & Elix 2016; Elix & MacCarthy 2018; McCarthy & Elix 2020, 2021). As listed in the Census of Plants of the ACT v. 4.1 (30 August 2019), 521 lichen species and infraspecific taxa occur in the territory, many of which were microlichens. Lichens occur in various ecosystems, from the dry soils of open *Eucalyptus* woodlands to exposed alpine rocky outcrops in Namadgi. Macrolichen genera well represented in the region are *Xanthoparmelia* and *Cladonia*, and diverse microlichen genera including *Buellia* and *Lecanora*. For lichen species identification, various keys and monographs are available, either as part of the volumes of the Flora of Australia (ABRS, Canberra and CSIRO, Melbourne) or as journal or online publications (e.g. Elix *et al.* 2017 for buellioid taxa; McCarthy 2012 for *Verrucaria*; McCarthy *et al.* 2020 for *Rhizocarpon*). A lichen checklist for Australia and its island territories is also available online (McCarthy 2020). In addition to high quality morphological data, chemistry data are available for a large number of species and specimens thanks to the work of Elix and others (e.g. Archer & Elix 1999; Elix 2009; Elix *et al.* 2009).

Although morphological and chemical data are available for many Australian lichens, including microlichens from the ACT, DNA sequence data is still sparse. Few molecular revisions have either targeted (e.g. Crespo *et al.* 2010; Lumbsch *et al.* 2010; Gueidan & Elix 2022) or included (e.g. Leavitt *et al.* 2018; Barcenas-Peña *et al.* 2021) Australian taxa, but most genera have not yet been the subject of molecular work. Well curated lichen material stored in Australian herbaria could however be used to produce such data, as methods to generate sequences from herbarium specimens are becoming more and more effective. These methods include protocols to obtain sequences from old herbarium specimens (Sohrabi *et al.* 2010; Redchenko *et al.* 2012; Kistenich *et al.* 2019). For example, using Ion Torrent sequencing technology, sequence data were obtained from lichen specimens up to 150-year-old (Kistenich *et al.* 2019). These new methods also allow the processing of large number of specimens at once, including 96 lichen specimens in Gueidan *et al.* (2019) and 384 lichen specimens in Gueidan and Li (2022). The potential of using lichen herbarium specimens for molecular taxonomy have however not yet been fully realised in Australia.

Reference sequence databases are important resources for various fields of science, including systematics, ecology, biodiversity and eDNA research. For fungi, the marker ITS was chosen as the fungal universal barcode early on (Schoch et al. 2012), and several well curated databases now provide access to large numbers of well-curated and high-quality ITS sequences, including UNITE (Kõljalg *et al.* 2013; Nilsson *et al.* 2018) and RefSeq (Schoch *et al.* 2014; O'Leary *et al.* 2015). Large taxon gaps however still exist in these databases (Orok *et al.* 2012; Kõljalg *et al.* 2013; Crous *et al.* 2014; Nilsson *et al.* 2018), and dried and living fungal collections have been proposed as possible solutions to generating ITS sequences from large number of species, specimens or strains (Yahr *et al.* 2016; Gueidan *et al.* 2019; Gueidan & Li 2022; Crous *et al.* 2014).

Here, as a first step towards the development of an ACT microlichen sequence database, we use CANB specimens (including types) and both traditional and next generation long-read sequencing techniques to generate ITS sequences for microlichens from the ACT. These sequences are then used to create a preliminary version of a sequence database for molecular identification purpose. This preliminary database is tested on several un-identified specimens collected during the 2018 ACT BushBlitz expedition and challenges to both generating sequences for a reference database and using this database to identify specimens to the species level are discussed.

Methods

Taxon selection and digitisation

The Census of Plants of the Australian Capital Territory (ACT) v. 4.1 (30 August 2019) was used to generate a list of microlichens from the ACT (Suppl. file 1a). Numerous specimens collected from the ACT are available from CANB (5,819 specimens, including 56 types), and from other Australian herbaria. Species with foliose, fruticose and dimorphic (foliose and fruticose) thalli were removed from the 521 listed lichen species in the ACT. A total of 254 species and infraspecific taxa with crustose to squamulose thalli (and sometime small foliose) were selected (Suppl. file 1b). After updating the list with new records and species, synonymies, re-identifications, and new combinations (Suppl. file 1c), the list included 246 taxa. Specimens of these 246 taxa were searched for in the CANB herbarium, and 99% of those were represented by at least one specimen in CANB. When available and large and recent enough for DNA work (in this case, collected after 1960), specimens from the ACT or NSW were chosen in priority over specimens from other Australian states and territories or overseas material. Twelve taxa could not be sampled, either because of the lack of specimens or available specimens were unsuitable (Suppl. file 1d). For the remaining 234 taxa, suitable specimens could be found in CANB (Suppl. file 2). A total of 394 CANB specimens representing these 234 taxa (including some multiple collections of the same taxon) were digitised using an Aptus Leaf II 12 camera and the Capture One software.

DNA extraction, amplification and sequencing

Specimens were sampled under a stereomicroscope using either (1) sterile tweezers or (2) clean razor blades and weighing paper. The ITS sequences were generated using two different approaches. Most samples were sequenced with a long-read amplicon approach, as described in Gueidan and Li (2022). In short, lichen material was ground using Lysing Matrix A tubes (MP Biomedicals) and a Precellys tissue lyser (Bertin Instruments) and the DNA extracted with the Invisorb DNA Plant HTS 96 kits (Stratec Molecular). A two-step amplification was then conducted, first using the ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) primers tailed with the PacBio M13 primers (first step of amplification), then using the PacBio M13 primers with unique index combinations (second step of amplification). The PCR products were cleaned, normalised and pooled, then sent for sequencing to Macrogen (Seoul, Korea), where a SMRTbell 1–2Kb amplicon library was prepared. One SMART cell of a Sequel I (V3) platform (Pacific Biosciences) was used to sequence the pooled sample, which included other PCR products from this study for a total of 548 samples. ITS sequences were obtained for additional specimens using a phenol-chloroform DNA extraction modified from Zolan and Pukkila (1986), as described in Gueidan *et al.* (2007). The ITS barcode was amplified using ITS1F and ITS4 primers and sequences were generated using Sanger sequencing at Macrogen, as described in Gueidan and Elix (2022).

ITS sequence editing and sequence database building

Sanger sequences were assembled and edited using Sequencher v. 5.4.6 (Gene Codes Corporation). For PacBio sequences, a BAM file of Circular Consensus Sequences (CCSs) was provided by Macrogen. The data was demultiplexed using the lima command in SMRT Tools v. 7.0.1 (Pacific Biosciences). The files were then converted to FATSQ files using BAMTools v. 2.5.1 (Barnett *et al.* 2011). CCSs were denoised using DADA2 v. 1.14 (Callahan *et al.* 2016) as described in Gueidan and Li (2022). Resulting sequence variants were converted to FASTA files using BAMTools and each file subjected to a blastn query using BLAST+ v. 2.12.0 (Altschul *et al.* 1990;

Camacho *et al.* 2009) against the NCBI nt database using a max_target_seqs of 3. For samples for which sequence variants were generated for the target species, only the main sequence variant was included in the database. For samples for which no sequence variants were generated for the target species, CCSs were converted to FASTA and subjected to the same blastn query to identify target CCSs. For these samples, all available target and non-chimeric CCSs were used as entries in the sequence database. All available sequences were added to a FASTA file. The sequence database was built using the makeblastdb command using BLAST+ v. 2.13.0.

Testing the molecular identification of BushBlitz microlichen specimens

To test the utility of this preliminary version of the ACT microlichen sequence database, eleven herbarium accessions representing 27 unidentified individuals of microlichens collected during the 2018 ACT BushBlitz expedition were used here (Table 1). Together with a broader sampling of a total of 140 BushBlitz microlichen specimens, they were digitised, sampled, extracted and sequenced using the PacBio amplicon protocol as described above. For the digitisation, as several specimens comprised multiple individuals (ie, multiple species occurring on the one fragment of substrate), generated specimen images were edited with Photoshop (Adobe) to indicate the sampled areas (Fig. 1). For these selected test specimens, resulting sequence variants were then subjected to two blastn searches with BLAST+ v. 2.13.0, one using the NCBI nt database, the other our created custom ACT microlichen database. Sequence identity matches superior to 95% were then compared to potential morphological identification of their respective specimens. Leica stereo- and compound microscopes and chemistry spot test were used to confirm the match between morpho-chemical and molecular identifications.

Results

Out of the 394 specimens used to generate ITS sequences for the custom ACT microlichen database, sequence data were obtained for 375 specimens. Among these, generated sequences of 151 samples could be unambiguously matched to their corresponding target taxon because their BLAST result matched the target species or genus with 80% similarity or more (Suppl. file 2). These sequences were used to create the first version of the database. For all others, additional work will be required to identify if the unmatching generated sequences were the result of mis-identification, mis-sampling, unclear species boundaries or sample cross-contaminations. Combined with data available from GenBank and data generated using Sanger sequencing, the first version of the database includes 347 sequences, representing 151 specimens from 99 microlichen species found in the ACT. The ACT microlichen ITS sequence database v.1 is available at https://doi.org/10.25919/qzaq-4182 [CSIRO Data Access Portal]. Additionally, ITS sequences will be deposited in GenBank once confirmed by more detailed and group-focused molecular taxonomic studies, as carried out in Gueidan & Elix (2022).

This sequence database was used in parallel to the NCBI nt database to identify specimens collected during the 2018 ACT BushBlitz expedition. The results for eleven accessions are presented in Table 1. For each of these accessions, one to several lichen individuals were sampled, for a total of 27 samples. For 15 of these samples, the blast results matched the lichen samples found in the accession (ie, morphological identifications confirmed molecular identifications). For four samples, the blast results likely matched the sample but, due to the small size of the thallus or the absence of fruiting bodies, the morphological identification could not fully confirm the molecular identification. For four samples, the molecular identification did not match the morphological identification (the species identified with molecular data could not be found on the accession). For all other samples (4), the blast result was below 95% identity and therefore unlikely to represent a usable match.

Discussion

Here, we used lichen herbarium specimens kept at CANB to generate ITS sequences for microlichens from the ACT. In order to reduce cost and effort, we processed samples in batches of 96 samples and used multiplexing for sequencing. Although material availability was not an issue (ie, most ACT microlichen species had usable specimens in CANB), mostly thanks

Table 1. Blast results (species match and percentage identity) and morphological identifications for 27 samples obtained from eleven 2018 ACT BushBlitz accessions. Main sequence variants (SVs) were used for the NCBI nt and the custom database blast searches.

	Collection number	**Herbarium accession**	**SVs NCBI nt database blast result (% identity)**	**SVs custom database blast result (% identity)**	**Morphological confirmation**
1	CG2447a	CANB910330	*Trapelia placodioides* 95.8%	*Trapelia placodioides* 96.4%	*Trapelia placodioides* confirmed
2	CG2447b		*Verrucaria rosula* 92%	*Verrucaria nigrescens* 87.7%	match with low similarity (< 95%)
3	CG2447c		*Japewia tornoensis* 84%	*Lecidea fuscoatrula* 82.4%	match with low similarity (< 95%)
4	CG2495	CANB910378	*Ramboldia petraeoides* 97%	*Ramboldia petraeoides* 98.4%	*Ramboldia petraeoides* confirmed
5	CG2414	CANB910297	*Trapelia elacista* 92.3%	*Trapelia crystallifera* 100%	*Trapelia crystallifera* confirmed
6	CG2434a	CANB910317	*Trapelia elacista* 98.6%	*Trapelia atrocarpa* 99.1%	*Trapelia atrocarpa* confirmed
7	CG2434b		*Trapelia tristis* 90%	*Trapelia concentrica* 99.2%	*Trapelia concentrica* confirmed
8	CG2434c		*Verrucaria rosula* 93%	*Endocarpon helmsianum* 89%	match with low similarity (< 95%)
9	CG2434d		*Trapelia placodioides* 95.8%	*Trapelia placodioides* 96.4%	*Trapelia placodioides* confirmed
10	CG2482a	CANB910365	*Lecanora farinacea* 98%	*Lecanora farinacea* 99.4%	*Lecanora farinacea* confirmed
11	CG2482b		*Ramboldia petraeoides* 98%	*Ramboldia petraeoides* 98.9%	*Ramboldia petraeoides* confirmed
12	CG2428a	CANB910311	*Trapelia placodioides* 95.4%	*Trapelia placodioides* 95.2%	*Trapelia placodioides* confirmed
13	CG2428b		*Trapeliopsis granulosa* 96%	*Trapeliopsis granulosa* 97.8%	mismatch, species not seen
14	CG2428c		*Buellia subarmeniaca* 92%	*Buellia halonia* 86%	match with low similarity (< 95%)
15	CG2433a	CANB910316	*Caloplaca* sp. 88%	*Caloplaca arandensis* 99.1%	mismatch, *Caloplaca* but not *C. arandensis*
16	CG2433b		*Trapelia lilacea* 93.9%	*Trapelia pruinosa* 99.1%	*Trapelia pruinosa* likely (apothecia not seen)
17	CG2433c		*Rhizocarpon nidificum* 99%	*Rhizocarpon geographicum* 99.6%	*Rhizocarpon geographicum* likely (apothecia not seen)
18	CG2433d		*Aspicilia epiglypta* 90%	*Aspicilia cinerea* 99.7%	*Aspicilia cinerea* confirmed
19	CG2445a	CANB910328	*Ramboldia petraeoides* 97%	*Ramboldia petraeoides* 98.3%	mismatch, species not seen
20	CG2445b		*Trapelia placodioides* 95.8%	*Trapelia placodioides* 96.4%	*Trapelia placodioides* confirmed
21	CG2476a	CANB910359	*Rhizocarpon smaragdulum* 96%	*Rhizocarpon vigilans* 94.2%	mismatch, *Rhizocarpon* but not *R. vigilans*
22	CG2476b		*Ramboldia petraeoides* 97%	*Ramboldia petraeoides* 98.2%	*Ramboldia petraeoides* confirmed
23	CG2423a	CANB910306	*Ramboldia petraeoides* 98%	*Ramboldia petraeoides* 98.6%	*Ramboldia petraeoides* confirmed
24	CG2423b		*Rhizocarpon badioatrum* 94%	*Rhizocarpon bicolor* 99.1%	*Rhizocarpon bicolor* likely (apothecia not seen)
25	CG2423c		*Rhizocarpon geographicum* subsp. *frigidum* 97%	*Rhizocarpon geographicum* 99.8%	*Rhizocarpon geographicum* likely (apothecia not seen)
26	CG2523a	CANB918145	*Lecanora farinacea* 100%	*Lecanora farinacea* 97.9%	*Lecanora farinacea* confirmed
27	CG2523b		*Ramboldia petraeoides* 98%	*Ramboldia petraeoides* 98.8%	*Ramboldia petraeoides* confirmed

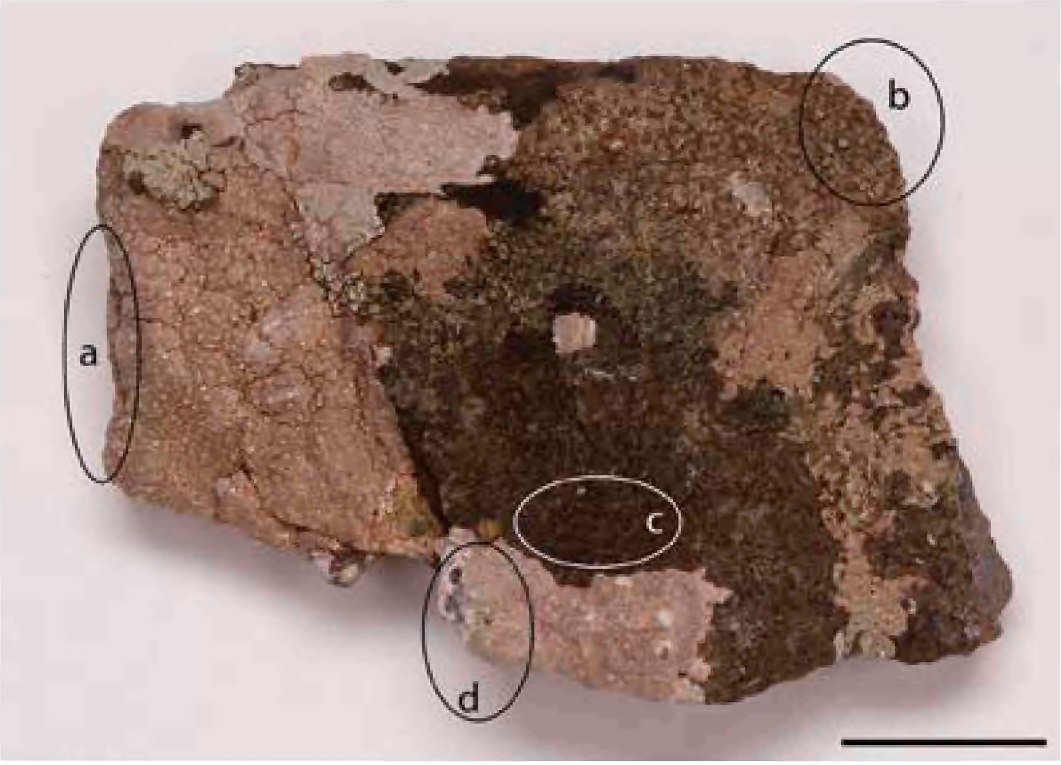

Figure 1. Annotated photograph of the 2018 ACT BushBlitz specimen CANB910317, showing the four sampled lichen thalli. The areas sampled for DNA extractions were encircled and labelled. The presence of three species of *Trapelia* (a. *T. atrocarpa*; b. *T. concentrica*; d. *T. placodioides*) was confirmed by molecular and morphological data. The presence of a species of Verrucariaceae was suggested by both molecular and morphological data, but with no close species match. Scale bar = 1 cm.

to the legacy collections of ACT-based lichenologists, other challenges were met, which are discussed below.

First, reliability in the available herbarium material is critical for building reference sequences. Although most used CANB specimens were accurately identified, some appeared to be mis-identified and led to unmatching molecular results. More importantly, in addition to mis-identification, is the issue of unclear species boundaries. For Australian lichens, most species boundaries are based on morpho-chemical data, with no molecular data available for confirming, for example, if a Northern hemisphere species indeed occurs in Australia or if this taxon should be described as new Australian-endemic species. Similarly, molecular data is critical to draw attention to morphologically similar but phylogenetically divergent species (convergent species), or species with particularly wide infraspecific variability. As a result, unless a lichen group has already been revised using both morpho-chemical and molecular data, the direct use of herbarium specimens to generate ITS sequences that reliably and accurately represent species is not realistic. Morpho-chemical and molecular revisions focussing on particular genera, subgenera or group of species, such as the one recently done on *Trapelia* (Gueidan & Elix 2022), will have to be done in parallel to specimen mass parallel sequencing, to clarify, or at least start clarifying, species boundaries. This will be a significant endeavour, which will require time and taxonomic expertise.

Second, reliability in the laboratory processes to generate the data is also critical to building reference sequences. Here, to reduce cost and time, a mass parallel/multiplexing sequencing approach was used, allowing to process large numbers of samples at once. This approach started with the sampling of material from a large number of herbarium specimens, a tedious process involving the removal of material from the substrate with a tweezer or a razor blade under a stereomicroscope. As microlichens often co-occur with other species on the same fragment of substrate, and some of these species are morphologically very similar, mis-sampling has also been the cause of mis-matching ITS sequences in this study. Additionally, even if the correct lichen thallus is sampled, if any material of neighbouring species is accidentally sampled and then preferentially amplified, sequencing can result in mis-matching sequences. To make things more difficult, lichen thalli harbor a high diversity of associated fungi, which will also be sequenced in the same process. For lichens, these are major issues which have to be added to the more common issues of sample cross-contamination (contamination between wells during the amplification steps) and index cross-talk (index mis-assignment after mass parallel sequencing). As a result, the generation of ITS sequences for lichens is not a straightforward process and will necessitate significant efforts of manual curation of the data. This manual data curation will make the application of automated laboratory and bioinformatic pipelines particularly challenging for this group of organisms.

As for the molecular identification of specimens using the generated reference sequence database, the same issues apply. Resulting sequences include at best the sequence of the target taxon, but also sequences of neighbouring species, associated lichen-inhabiting fungi, and possible cross-contaminants. It is therefore again not a straightforward process and will require input from a taxonomic expert. Fully automated molecular identification processes will therefore be difficult to apply at this stage. The reliability of molecular identifications will also depend on solid morpho-chemical and molecular revisions of Australian lichen taxa. These revisions will require time and taxonomic expertise, but once species concepts are clarified, reference sequence databases are a powerful tool to accelerate and confirm species identifications. This was demonstrated here by the higher BLAST identity percentages obtained with our ACT microlichen custom sequence database as compared to the percentages obtained with the current NCBI nt database. It was also evident for the recently revised Australian *Trapelia* species (Gueidan & Elix 2022): all unidentified *Trapelia* material collected during the 2018 ACT BushBlitz expedition were correctly identified to the species level thanks to their ITS sequences and our newly generated sequence database for ACT microlichens.

Whether carried out in the field or in the laboratory, species identifications using molecular sequences is a powerful tool to the study of taxonomy, ecology and biodiversity, especially for organisms with limited diagnostic features. It however relies on the development of high-quality reference sequence databases. Although generating these references from collection specimens may be a straightforward process for some organisms, it has some challenges for lichens, mostly due to unclear species concepts, but also to other issues such as mis-identification, mis-sampling and cross-contamination. As a result, initial molecular taxonomic revisions of Australian material from all the main lichen genera will be necessary before mass parallel sequencing of herbarium specimens can be used to generate reliable reference sequence databases.

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Supplementary file 1. Lichenised fungal species and infraspecific taxa from the ACT, as listed in the Census of Plants of the Australian Capital Territory v. 4.1 (30 August 2019). There are four worksheet tabs:

a. list of all species as published in v. 4.1;
b. list of all ACT microlichen species;
c. list of all ACT microlichen species after name update;
d. list of ACT microlichen species for which specimens were available in CANB.

Supplementary file 2. List of specimens used to generate ITS sequences for the ACT microlichen ITS sequence database, with voucher information.

Supplementary files 1 and 2 are appended to the online listing of this paper, accessible via:
https://www.rbg.vic.gov.au/science/journal/

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A sorediate addition to the genus *Megaloblastenia*

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Abstract

The epiphytic species, *Megaloblastenia sorediata* Kantvilas sp. nov., is described from the wet forests of Tasmania and New South Wales; its preferred host is the understorey tree *Pomaderris apetala*. The newly described species is distinguished from the widespread *M. marginiflexa* (Hook.f. & Taylor) Sipman chiefly on the basis of having soredia, although its apothecia, asci and ascospores are also marginally smaller. A key to the three species of *Megaloblastenia* is presented, and the application of soredia as a taxonomic character is discussed briefly.

Keywords: Australia, lichens, species pairs, Tasmania, taxonomy

Introduction

Megaloblastenia Sipman is a small genus of three taxa, restricted to oceanic climates in temperate to tropical latitudes of Australasia and South America, and typically occurring as epiphytes in moist forests. It is characterised by a crustose, mostly ecorticate thallus containing *Dictyochloropsis* as the photobiont, biatorine to lecideine, relatively large apothecia, a non-inspersed hymenium, clavate, 8-spored asci with an intensely KI+ blue outer coat, a well-developed KI+ blue tholus lacking internal differentiation, and sometimes with a short, conical, ocular chamber (*Megalospora*-type: see Kantvilas & Lumbsch 2012, Fig. 1D), slender, branched and anastomosing paraphyses, and hyaline, bicellular ascospores with thickened polar and septal walls and the locules connected by a narrow channel (polaribilocular: Kantvilas & Lumbsch 2012, Figs 1E-F). It is classified in the family Megalosporaceae (Teloschistales) together with the genus *Megalospora* Meyen, which, although superficially similar, differs by having a hymenium densely inspersed with oil droplets, and transversely septate or muriform ascospores. Thallus chemistry in both genera is relatively simple and includes zeorin, together with either pannarin or usnic acid.

The family Megalosporaceae was comprehensively monographed by Sipman (1983) who established the framework of characters, chiefly based on ascospores, by which the species are defined. Since that time there have been further new taxa and new records of *Megalospora* for Australia (including Tasmania) by Allen *et al.* (2001), Elix (2009), Hafellner *et al.* (1989), Kantvilas

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(1994, 2008, 2018), Sipman (1986) and Vězda (1989), bringing the total to 19 (McCarthy 2020). *Megaloblastenia*, however, has continued to comprise only the three taxa recognised by Sipman (1983), two of which occur in Australia. However, its distinct and separate status has been confirmed by DNA-sequence data (Gaya *et al.* 2012; Kantvilas & Lumbsch 2012). Here, a further species from the wet forests of Tasmania and south-eastern mainland Australia is described and illustrated.

Methods

The study is based mainly on specimens housed in the Tasmanian Herbarium (HO) and on field observations made by the author. Morphological and anatomical investigations were undertaken on hand-cut sections of the thallus and apothecia using standard methods, reagents and stains: water, 10% KOH (K), Lactophenol Cotton Blue, ammoniacal erythrosin and Lugols Iodine (I). Amyloid reactions where sections were pre-treated in K, rinsed in water and then mounted in I are described in the text as KI. Calcium oxalate was detected by eluting thin sections with 20% H_2SO_4. Measurements of ascospores are presented in the format 5th percentile–*average*–95th percentile, with outlying values in parentheses and *n* indicating the number of observations. Routine chemical analysis was undertaken using standard methods (Orange *et al.* 2010); solvent A was the preferred medium.

Taxonomy

***Megaloblastenia sorediata* Kantvilas, sp. nov.**

Mycobank No.: MB 846876

Similar to *M. marginiflexa* (Hook.f. & Taylor) Sipman, from which it differs by the convex to excavate soralia, 1–2 mm wide, the smaller asci, 80–110 x 12–26 µm, and smaller ascospores, 20–34 x 10–20 µm.

***Type*:** Australia, Tasmania: Stony Head MTA, Ryans Hill, SE of summit, 41°01′05″S 147°01′43″E, 210 m, on *Pomaderris apetala* in wet sclerophyll forest, 4 November 2020, *G. Kantvilas 212/20* (holo - HO; iso - CANB)

Thallus pale grey or greenish grey, ecorticate, smooth to rimose, rarely verruculose, sorediate, lacking calcium oxalate, forming patches to *c.* 10 cm wide; soralia discrete, at first punctiform, at length convex or excavate, 1–1.5(–2) mm wide, occasionally becoming confluent; soredia farinose to granular, whitish or ± concolourous with the thallus. *Photobiont Dictyochloropsis*, with ± globose cells 6–12 µm wide. *Apothecia* biatorine, sessile, basally constricted, 0.8–2.5(–3) mm diam.; disc plane, pale to dark reddish brown, mostly with a thin, sometimes patchy, bluish grey pruina; proper exciple prominent and persistent, entire, pale to deep red-brown to dark brown, usually darkest at the rim, in section cupulate, 90–200 µm thick, pale yellow-brown to dark brown, darkest along the inner edge and especially so beneath the hypothecium, composed of tightly packed, radiating, branched and anastomosing, conglutinated hyphae, inspersed with calcium oxalate crystals. *Hypothecium* 60–90 µm thick, hyaline to yellowish brown in the lower part, usually inspersed with oil droplets. *Hymenium* 90–140 µm thick, hyaline, not inspersed, I+ blue, KI+ blue, overlain by a yellow-brown to red-brown epithecial layer to *c.* 10–15 µm thick. *Asci* clavate, 80–110 x 12–26 µm, 6–8-spored, of the *Megalospora*-type. *Ascospores* hyaline, ellipsoid, non-halonate, polaribilocular, (20–)22–*26.0*–30(–34) x (10–)11–*14.3*–18(–20) µm (*n* = 60), with the locules connected by a narrow channel. *Pycnidia* uncommon, immersed in small verrucae *c.* 0.2 mm wide; conidia bacilliform, 3-5 x 0.5 µm. Chemistry: pannarin, zeorin; thallus K–, KC-, C– P+ orange, UV- or dull grey. Figs 1A-B.

***Etymology*:** The specific epithet refers to the thallus being sorediate, a character that distinguishes it from the other species of the genus.

***Distribution and ecology*:** The ecological observations presented here are based on fieldwork by the author in Tasmania, where the newly described species is common. There, *Megaloblastenia sorediata* is widespread, locally abundant and most commonly collected in lowland wet

Key to *Megaloblastenia* in Australasia

1 Thallus pale yellowish, P– (containing usnic acid and zeorin); apothecia entirely black and epruinose; Tas, NZ.. ***M. flavidoatra***

1: Thallus grey, P+ orange (containing pannarin and zeorin); apothecia usually with a pale, reddish brown, thinly grey-pruinose disc and yellowish to dark brown margin.. 2

2 Thallus sorediate; Tas, NSW.. ***M. sorediata***

2: Thallus lacking soredia; Tas, Vic, NSW, NZ.. ***M. marginiflexa***

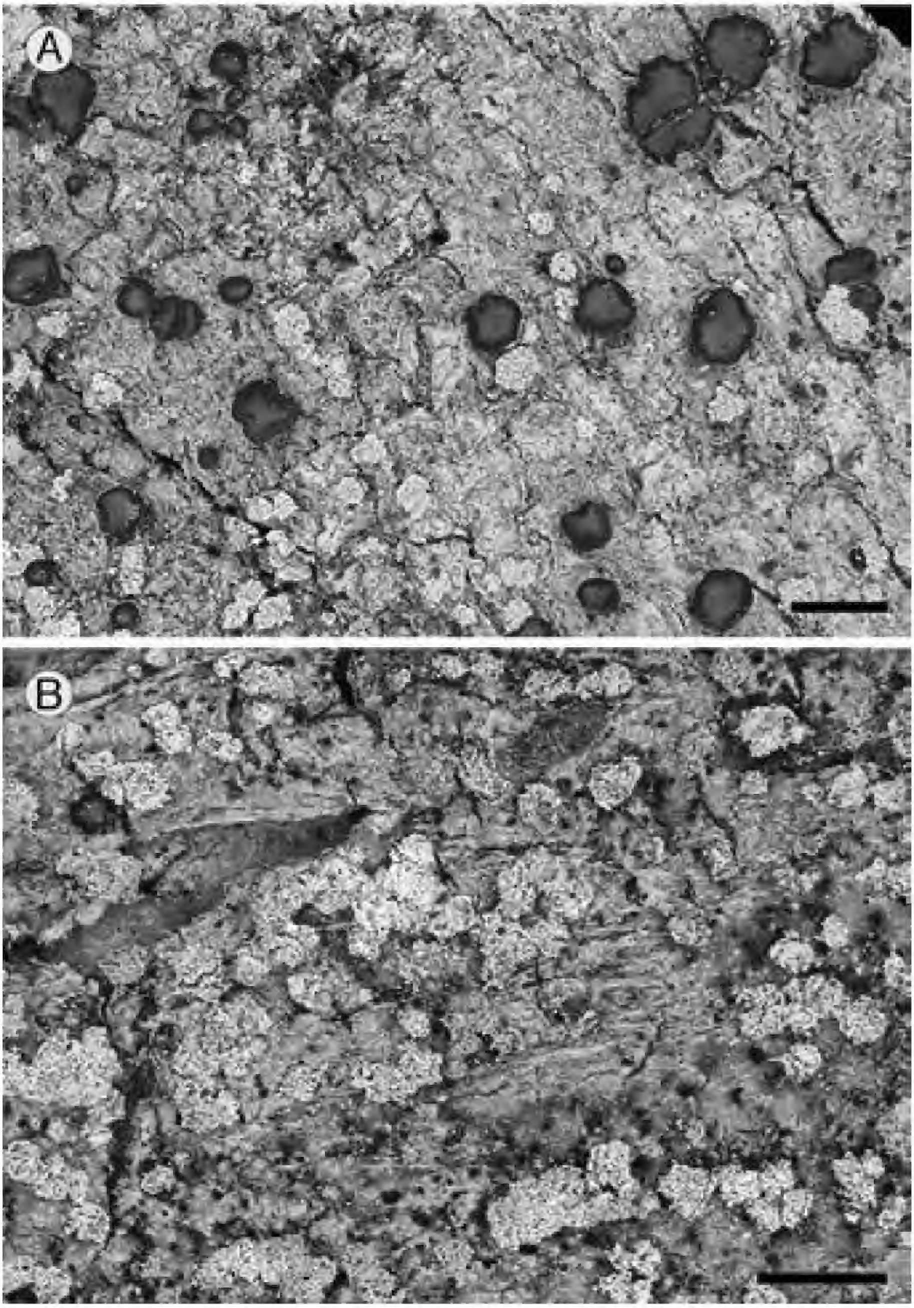

Figure 1. *Megaloblastenia sorediata* habit. **A.** Fertile thallus with discrete, roundish soralia and abundant apothecia (holotype). **B.** Sorediate, sterile thallus dominated by soralia with coarse, granular soredia (*Kantvilas 317/21*). Scale = 2 mm.

sclerophyll forest or tall, coastal scrub where it particularly favours the bark of *Pomaderris apetala*. However, it has also been found in such vegetation communities on other trees with a similar smooth bark, such as *Hakea lissosperma*, *Notelaea ligustrina* and *Banksia marginata*, or on the wood of *Bedfordia salicina*. In these habitats, it is part of a rich assemblage of chiefly-crustose lichens, with the dominant species being *Megalaria melaloma* (C.Knight) Kantvilas, *M. melanotropa* (Nyl.) D.J.Galloway, *Pyrenula ravenellii* (Tuck.) R.C.Harris, *Sarrameana albidoplumbea* (Hook.f. & Taylor) Farkas, *Strigula albicascens* (Nyl.) R.C.Harris and *Thelotrema lepadinum* (Ach.) Ach. Also invariably present in these epiphytic communities is the closely related *Megaloblastenia marginiflexa*, and it is because it grows so closely associated with *M. sorediata* that the latter was overlooked as distinct for so long (see discussion below). Although the two species co-occur in this habitat, *M. marginiflexa* is generally more widespread and, in Tasmania, extends across most of the island and is also found in *Nothofagus*-dominated cool temperate rainforest. *Megaloblastenia sorediata* also occurs in *Melaleuca ericifolia*-dominated swampy woodland, a vegetation type remarkable for its concentration of lichens of high conservation significance, such as *Bactrospora metabola* (Nyl.) Egea & Torrente, *B. paludicola* Kantvilas, *Coniocarpon cinnabarinum* DC., *Enterographa micrographa* (Nyl.) Redinger, *Haematomma sorediatum* R.W.Rogers and *Pseudocyphellaria aurata* (Ach.) Vain. (Baker *et al.* 2021; de Salas *et al.*, in press). Sipman (1983) clearly saw sorediate material, which he included under *M. marginiflexa*, but makes no specific mention of its provenance.

Although no specimens from Victoria have been seen, *M. sorediata* can confidently be expected to occur there due to the abundance of the appropriate wet forest habitat. The same applies to the coastal ranges of New South Wales, from where a single specimen has been collected. The author has not encountered any sorediate specimens of *Megaloblastenia* in New Zealand although the species could well occur there.

***Selected specimens examined*: TASMANIA:** Three Thumbs, 42°36′S 147°52′E, 350 m, 4.vii. 1987, *G. Kantvilas s.n.* (HO); Deadmans Bay, 43°32′S 146°30′E, 5 m, 17.i.1987, *A. Moscal 14096a* (HO); Denium Hill, Robbins Island Track, 40°45′S 144°53′E, 5 m, 10.xii.1993, *G. Kantvilas 140/93 & J. Elix* (HO); High Yellow Bluff, 42°56′S 147°59′E, 250 m, 27.xii.2000, *G. Kantvilas 551/00* (HO); Sandspit River, 42°43′S 147°51′E, 170 m, 16.vi.2010, *G. Kantvilas 102/10* (F, HO); Lichen Hill, 43°04′S 147°56′E, 570 m, 10.viii.2010, *G. Kantvilas 120/10* (HO); south-western slopes of Mt Fortescue, 43°10′S 147°58′E, 440 m, 6.iv.2012, *G. Kantvilas 269/12* (HO, MEL); Hellfire Bluff, 42°44′S 147°55′E, 150 m, 13.ix.2014, *G. Kantvilas 377/14* (HO, UPS); track to St Columba Falls, 41°19′S 147°55′E, 300 m, 27.vi.2020, *G. Kantvilas 107/20* (HO); Stony Head MTA, Quarry Road, 41°02′26″S 146°59′40″E, 50 m, 19.iii/2021, *G. Kantvilas 88/21* (HO); *c.* 7 km N of Branxholm, 41°06′S 147°44′E, 220 m, 10.ix.2022, *G. Kantvilas 470/22* (HO). **NEW SOUTH WALES:** Brown Mountain, Rutherfords Creek, 36°35′22″S 149°26′44″E, 815 m, 17.iv.2008, *G. Kantvilas 106/08, J. Elix & P. McCarthy* (HO, NSW).

Discussion

Lichens offer many examples where there are exclusively sexually reproducing entities and closely related asexually reproducing ones, with or without apothecia. However, how to deal with these taxonomically is somewhat controversial. Du Rietz (1924) noted that sorediate and esorediate morphs of a lichen often displayed different geographical distributions and ecological preferences. He elected to recognise them as species and coined the term “Artenpaar”, a concept developed further by Poelt (1970, 1972). Species pairs were generally adopted with enthusiasm for several lichen groups, such as in the Parmeliaceae and *Pseudocyphellaria*, and have stood the test of time and further investigations. At the same time, other lichenologists, for example Tehler (1982) in his studies of the Roccellaceae, readily accepted sorediate and esorediate morphs within a single species. The concept of species pairs is discussed extensively by Mattson & Lumbsch (1989) who offer guidelines for their taxonomic evaluation but essentially recommend that each case be treated on its merits. In the Australian biota, there are clear examples where the species pair idea has been applied and is generally accepted [e.g. *Menegazzia subbullata* P.James & Kantvilas (sorediate) versus *M. elongata* P.James (fertile only)]. At the same time, there are also cases, such as the widespread *Trapeliopsis colensoi* (C.Bab.) Gotth.Schneid., where exclusively sorediate forms, exclusively fertile forms, and sorediate forms with abundant apothecia all co-occur and are considered conspecific.

It is largely because of this controversy that the author has, for a considerable time, been reluctant to uncritically recognise the sorediate morphs of *M. marginiflexa* as distinct, even as field observations across several decades were suggesting otherwise. In the herbarium, it is easy to overlook the sorediate forms, especially as collectors inevitably strive to collect fertile material and apothecia on the sorediate morph tend to be fewer and smaller. However, in the field, the distinction becomes more apparent and, where both are present, it becomes obvious that there are two distinct entities in play: the exclusively fertile, esorediate one and an unequivocally sorediate one. An attempt to explore the relationship between the two morphs using molecular methods was made by Kantvilas & Lumbsch (2012). However, obtaining usable DNA proved difficult, despite the abundance of fresh material available, and the result, although placing sorediate and non-sorediate morphs in separate clades, was not conclusive.

Detailed anatomical study revealed further, albeit subtle differences between the two entities that support their recognition at species rank. *Megaloblastenia sorediata* is generally smaller in all its parts: the apothecia are rarely more than 2.5 mm wide, whereas in *M. marginiflexa* they are commonly as wide as 3.5 mm, and may even be as wide as 5 mm; the asci in *M. marginiflexa* are 110–140 x 20–35 µm, whereas in *M. sorediata* they are 80–110 x 12–26 µm. Critically there is also a difference in ascospore size with those of *M. marginiflexa* being (26–)29–*36.0*-42(–44) x (14–)15–*18.9*–24(–25) µm (*n* = 60), compared to (20–)22–*26.0*–30(–34) x (10–)11–*14.3*–18(–20) µm in *M. sorediata*.

A possible parallel example of a sorediate-esorediate species pair in the Megalosporaceae is seen in *M. subtuberculosa* (C.Knight) Sipman (sorediate, rarely fertile) and *M. melanodermia* (Müll.Arg.) Zahlbr. (exclusively fertile). In the former, the ascospores are 30–*40.4*-50(–56) x 19–*24.9*–31(–34) µm (this study), whereas in the latter, they are (32-)36-*42.0*-48(-54) x 20–*24.0*–30 µm (Kantvilas 1994). In this case, there is no significant difference in ascospore size, even though the notion that the two species are distinct has never been in dispute.

Acknowledgements

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Conservation and genomic diversity of a rare tree, *Eucalyptus imlayensis* (Myrtaceae), regenerating after wildfire

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Abstract

Rare species confined to small populations are frequently targeted for conservation because they are prone to reduced fitness stemming from inbreeding depression, loss of diversity and genetic drift resulting in an increased extinction risk. In this study, we used genome-wide scans to quantify clonal diversity and plant relatedness in the only known population of *Eucalyptus imlayensis* which was regenerating after wildfire burnt the entire population in summer 2019/2020. Samples taken from resprouting lignotubers were used to generate a dataset of single nucleotide polymorphisms (SNPs) and contained 406 loci across 51 stems and 5 technical replicates. Clonality was found to be extensive, with the entire population of *E. imlayensis* composed of only 17 distinct genotypes. Ten genotypes were confirmed as clonal with the remaining 7 genotypes each recovered only once. Kinship analysis revealed two unique genotypes with a first-degree relationship to one of the clonal genotypes, and five samples with a second-degree or beyond relationship to any clonal groups. This information will form the basis for the development of an *ex situ* collection and manipulated crosses to produce diverse seed for population enhancement, as well as to establish a seed store for conservation.

Keywords: clonality, single nucleotide polymorphism, conservation genetics, relatedness

Introduction

Fire has been a significant evolutionary force for much of the Australian flora, with adaptations that include the ability to re-sprout from below-ground meristems and soil-stored seedbanks. Such adaptations enable some species to withstand the wholesale consumption of the plants above-ground by fire (Burrows 2013). On a global scale, climate change has precipitated shifts in fire regimes, resulting in longer fire seasons with more frequent and more destructive fires (Jones *et al.* 2022). The extent and severity of these fires impact areas not previously exposed to frequent or intense burning

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(Filkov *et al.* 2020). This general trend is reflected in the fire history of south-eastern Australia over recent decades (Dutta *et al.* 2016; Boer *et al.* 2020), most notably in 2019/2020, where fires burnt more than 7 million ha of dry sclerophyll forest dominated by re-sprouting eucalypts (Filkov *et al.* 2020; Gibson & Hislop 2022). The evolution of re-sprouting is linked to the rise of flammable sclerophyll biomes in Australia from the early Cenozoic (60–62 mya), and in eucalypts the anatomy of epicormic and basal sprouting from lignotubers has been preserved despite changes to habitat and response to fire (Crisp *et al.* 2011).

Mount Imlay National Park (Figure 1), in south-eastern New South Wales (NSW), incorporates Mount Imlay, an isolated and culturally significant peak that rises to 886 m above sea level (a.s.l.) and which is known to local peoples as Balawan (Department of Agriculture, Water and the Environment 2022a). Mount Imlay and the surrounding park were burnt extensively when a wildfire moved rapidly from the south on New Year's Eve 2019, affecting typically moist vegetation that had become drier during several preceding years of drought. Mount Imlay is considered an Asset of Intergenerational Significance for two endemic plants species, *Eucalyptus imlayensis* Crisp & Brooker and *Hibbertia circinata* K.L.McDougall & G.T.Wright, under the amended NSW *National Parks and Wildlife Act 1974*. There is one other endemic plant species on Mt Imlay (*Boronia imlayensis* Duretto) and several other significant plant species, most of which are threatened by the pathogen *Phytophthora cinnamomi* (McDougall *et al.* 2023).

Mallees such as *Eucalyptus imlayensis* are usually found in semi-arid, fire-prone regions of southern Australia but in high rainfall areas, they are often confined to 'difficult' sites characterised by shallow soils over bedrock (Lacey & Johnston 1990). The only known population of *E. imlayensis* occurs just below the summit on the eastern face of Mt Imlay at 850 m a.s.l. and is confined to a site with rocky, shallow soils. The area receives an average annual rainfall of 750 mm. Plants have a characteristic mallee growth form with multiple stems to 7 m tall originating from a lignotuber, a dense canopy and smooth bark that sheds in ribbons (Crisp & Brooker 1980).

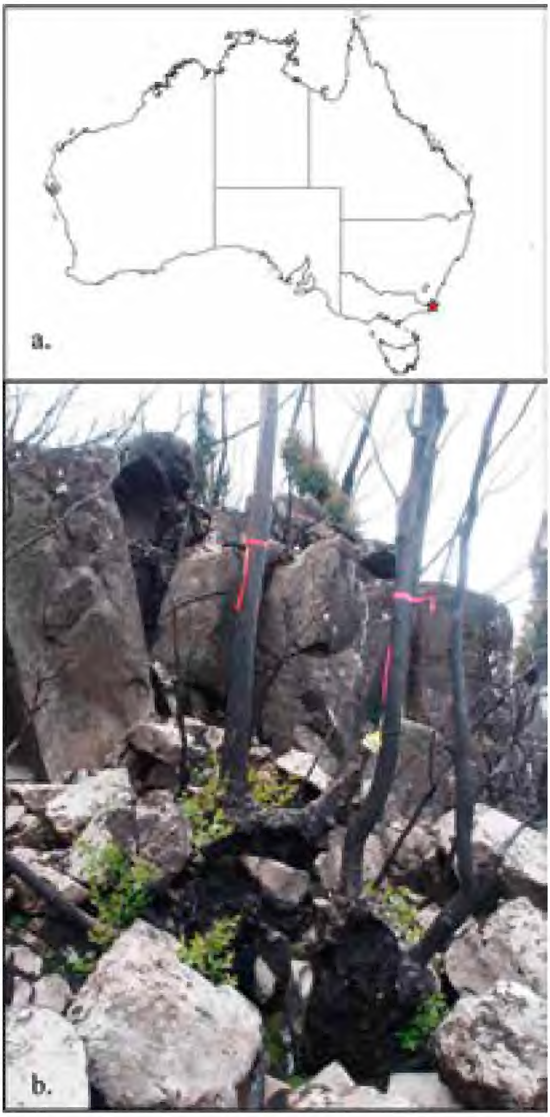

Figure 1. a. Location of Mt Imlay in south-eastern New South Wales (asterisk) where the only population of *Eucalyptus imlayensis* is situated, and b. re-sprouting lignotubers nestled amongst granite boulders as recorded on 6 November 2020 approximately 10 months after above-ground stems were killed by fire (image: G. Wright).

In 2022, *Eucalyptus imlayensis* was identified as one of 100 priority threatened species in the Commonwealth of Australia's Threatened Species Strategy Action Plan 2022-2032 (Department of Climate Change, Energy, the Environment and Water 2022). It is listed as Critically Endangered under the New South Wales *Biodiversity Conservation Act 2016* and the federal *Environment Protection and Biodiversity Conservation Act 1999* (NSW Government 2016, AWE 1999) and managed under the

NSW government Saving Our Species (SOS) program. The high-intensity wildfire of 2019/2020 consumed all vegetation within the habitat of *E. imlayensis* and highlighted the vulnerability of this highly restricted species to a single high intensity fire. Seed production from fire-impacted plants is not expected to commence for at least 10 years, based on the absence of flowers on seed-grown plants planted *in situ* in 2011. As a result, *E. imlayensis* is listed as requiring urgent post-fire assessment (Gallagher 2020), with the establishment of an *ex situ* conservation collection developed from asexual material (cuttings) part of urgent conservation action post-fire (DPE 2022).

Due to its very small area of occupancy (0.3 ha), *Eucalyptus imlayensis* has been of high conservation concern since it was first described (Crisp & Brooker 1980). Extensive surveys were undertaken between 1998 and 2003 to monitor the health and size of the population which was estimated to comprise approximately 80 multi-stemmed plants (James & McDougall 2007). Separate lignotubers were considered to represent distinct plants, each of which were tagged and mapped on site. No juvenile or seedling plants have been observed within the population in the past 20 years, and seed production is limited and sporadic. Genetic studies in 2007 identified five genotypes from 27 *E. imlayensis* samples analysed, indicating fewer genetic individuals than lignotubers genotyped and a high level of clonality (James & McDougall 2007). However, it was recognised at the time that there may be additional undetected plants because the steep terrain and dense growth of *Leptospermum scoparium* limits accessibility at the site (James & McDougall 2007). The absence of dense vegetation in the post-fire landscape aided surveys and accurate mapping of the population in May 2020. Despite above-ground stems being killed, most *E. imlayensis* adult plants survived the wildfire and were re-sprouting from lignotubers (Figure 1).

Identifying the genetic signature of plants and populations and the relationships between them have long been recognised as vital for genetically representative seed banking, *ex situ* conservation, and conservation-focussed breeding programs (Griffith *et al.* 2021). In this study, a comprehensive genetic assessment of all surviving plants within the population of *Eucalyptus imlayensis* was undertaken. Genome-wide scans were utilised to identify the extent of clonality, kinship and genotypic diversity. This information provides the basis for future conservation efforts enabling reproductive variability and genotype survival to be tracked over time. Here, we aim to 1) determine extent of clonality in the population; 2) identify the number and size of clones; 3) map the spatial distribution of genotypes; and 4) assess the relatedness between genotypes to develop a breeding strategy for *ex situ* seed production and potential population augmentation.

Methods

Fieldwork and sampling

Field work was undertaken by the NSW Department of Planning and Environment, Biodiversity Conservation staff in November 2020, as part of the annual SOS monitoring program. During this survey work, 48 *Eucalyptus imlayensis* plants were re-tagged and geolocated. A 'plant' was defined as a group of stems that appeared to belong to the same lignotuber, and a 'stem' was defined as a single shoot or trunk from a lignotuber. The removal of vegetation by the fire 11 months prior to the survey enabled a more accurate assessment of the number of plants because previously unseen associations between lignotubers were visible and reduced the plant count from the 80 estimated in 2006 (James & McDougall 2007); some plants had also died since 2006. Not all resprouting plants could be reconciled with previously mapped plants because tags were burnt, and not all known plants could be found post-fire. However, the ability to locate all re-sprouting plants enabled the first accurate count and genetic assessment of the population.

Although most re-sprouting lignotubers surveyed in 2020 were presumed to correspond to previously tagged plants, a new number was assigned to each apparent individual to avoid confusion. It was not possible to determine visually whether stems were clonemates or belonged to distinct genets based on proximity in the field because physical connections between lignotubers may have been lost over time. All live stems were re-shooting from existing lignotubers with no evidence of newly recruited seedlings in the population. To identify the number of genets, assess relatedness between plants (genets) and identify clonemates (ramets), in

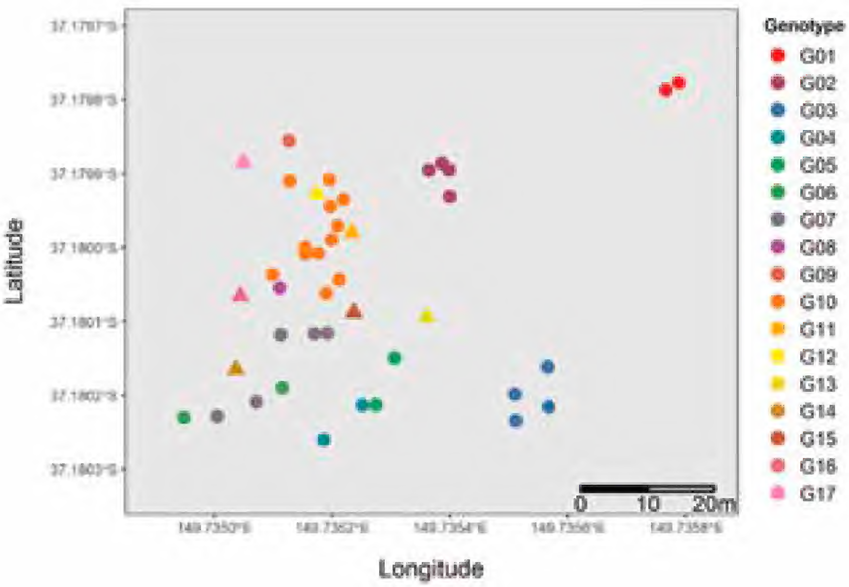

Figure 2. Site map showing spatial distribution of sampled *Eucalyptus imlayensis* stems within the site, colour-coded for genotype. Genotypes *G1–G10* were recovered multiple times and are represented by circles while *G11–G17* were recovered only once and are represented by triangles.

November 2020, leaf samples for genetic analysis were collected from one stem of each of the 48 newly tagged plants likely to be a mix of genets and ramets. The location of each sample was georeferenced with a handheld GPS. A duplicate sample was collected from each of three tagged plants from a second stem on the same lignotuber and within 1 m of the primary sample giving a total of 51 samples. The duplicate samples were annotated with 'a' to differentiate them from the paired sample of the same lignotuber or plant (*103,103a*; *115, 115a*; *117, 117a*). Samples were stored in silica gel and sent to Royal Botanic Gardens Victoria for preparation. The locations of samples used in the final analyses are shown in Figure 2.

Molecular data collection

Approximately 15 mg from each of the 51 leaf samples, plus 5 technical repeats (56 in total), were subsequently sent to a commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq™ analysis, which is a reduced representation sequencing method proven for use in eucalypts (Sansaloni *et al.* 2010; Kilian *et al.* 2012; Cruz *et al.* 2013). DNA was extracted at DArT™ using the Nucleo Mag Plant C-Bead kit (Machery Nagel, Germany) on a Tecan 100 platform per the manufacturer's protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and HpaII and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina HiSeq2500. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT™'s proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DarTsoft14), producing 18 427 binary SNPs from 55 samples as one individual did not yield sequences.

Molecular data analysis

Clonality was investigated within 55 samples made up of 47 *Eucalyptus imlayensis* plants from tagged lignotubers (no data from *eim124*), the paired samples from three different lignotubers, and 5 technical repeats, following the methods of Bradbury *et al.* (2021). Variation is known to occur among clonemates due to genotyping errors and somatic mutations and can lead to over-estimation of genotypic diversity. To minimise this, the average sequencing error rate in the unfiltered dataset (18.86 ±1.17 SD) was estimated by calculating the average genetic distance between known identical samples (our technical replicates) using the R package dartR (Gruber *et al.* 2018). To enable an accurate estimate of clonality, a threshold of genetic differentiation is typically applied to determine the degree of variation that is acceptable between ramets (Douhovnikoff & Dodd 2003; O'Brien *et al.* 2014). Therefore, a pairwise individual Euclidean genetic distance matrix was constructed with the expectation that distance values between genuinely distinct genotypes (genets) versus near-identical genotypes (ramets) should clearly differ. From our dataset, pairwise samples with a distance value ≤37.72 (i.e., twice the average error rate, to account for deviation from the mean) were considered ramets of a clonal genotype. The mean distance between clonal samples (ramets) of 24.85 (±5.89 S.D.) was clearly distinct from the mean distance among all other stems (91.71±12.15 S.D.), giving us high confidence in clone identification and a low risk of over-estimating genotypic diversity. From these analyses, we identified 17 genotypes with varying numbers of ramets recovered per genotype.

To verify the relationship of the clonal stems, the technical replicates were removed, data were filtered using the R packages dartR (Gruber *et al.* 2018), poppr (Kamvar *et al.* 2014, 2015) and SNPRelate (Zheng *et al.* 2012) in R (R Core Team 2019) to a locus and individual call rate of 85% and 100% (respectively), a reproducibility score of 1, a Hardy-Weinberg equilibrium with a 5% level of significance, a minor allele frequency greater than 5%, removal of monomorphic loci, and finally filtered on Hamming distance to remove potential paralogues. The resultant dataset contained 2980 SNPs across 50 individuals.

Pairwise genetic relatedness (kinship coefficient, ϕ) was calculated between individuals using the identity by descent maximum likelihood estimation method in the R package SNPRelate (Zheng *et al.* 2012; Manichaikul *et al.* 2010). Kinship values of 0.50 represent a monozygotic twin, ϕ=0.25 and higher indicate a first-degree relationship (full siblings, parent-child etc.) and ϕ=0.125-0.25 indicate a second-degree relationship (i.e., half-siblings, grandparent-grandchild etc). Unrelated pairs are expected to have values close to ϕ=0 (Manichaikul *et al.* 2010). A kinship threshold of k=0.45 was used to define ramets (Bragg *et al.* 2021).

To visualise relationships among plants and genotypes, a principal components analysis (PCA) was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011). PCA does not rely on an evolutionary model and is therefore free of the assumptions made by other clustering methods (Jombart *et al.* 2009). A neighbour-joining tree was plotted using the ape package in R (Paradis & Schliep 2019). The R packages pophelper (Francis 2017), ggplot2 (Wickham 2016) and FigTree v1.4.4 (Rambaut 2018) were used to visualise results, and individuals were mapped on to the site layout according to their genotype.

Finally, a third dataset was assembled using one representative of each genotype selected at random and filtered as described above. The dataset contained 3165 variable SNPs from 17 individuals. Individual heterozygosities and inbreeding coefficients were calculated for each genotype. A genomic relatedness matrix for the genotypes was calculated with the gl.grm function and plotted as a heatmap in dartR (Gruber *et al.* 2018). An individual genetic distance matrix (Euclidean) was produced in the R package dartR (Gruber *et al.* 2018) and a pairwise genetic relatedness (kinship coefficient, ϕ) was calculated between genotypes as described above, in the R package SNPRelate (Zheng *et al.* 2012; Manichaikul *et al.* 2010).

Results

Analyses on the unfiltered dataset identified 17 genotypes composed of ten recurrent (clonal) genotypes (*G01 – G10*) and seven genotypes (*G11– G17*) recovered only once (unique). Individual Euclidean genetic distances (GD) and kinship coefficients (ϕ) between the 17 *Eucalyptus imlayensis* genotypes were calculated based on 3165 SNPs and ranged from GD = 24-59 and ϕ = 0-0.37 (Table 1; *see also* Suppl. files 1 & 2). Within each clonal genotype, ϕ values among ramets ranged from 0.47 to 0.49 which is within the threshold of 0.50 expected for monozygotic twins (Suppl. file 2). Individual observed heterozygosity ranged from 0.221 (*G11*) to 0.474 (*G05*; *see* Suppl. file 3) and inbreeding coefficients ranged from -0.272 (*G05*) to 0.406 (*G11*).

Clonal genotypes ranged from having two distinct lignotubers (*G1*, *G8*) to having 13 lignotubers (*G10*) excluding duplicate samples. Ramets of each genotype are, on the whole, spatially clustered (Figure 2). Genotype *G10* occupies the largest area and appears to have become fragmented after expansion. Two unique plants, *G11* and *G12,* are located within the geographic spread of *G10* with kinship coefficients indicative of a

	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17
G01		0.08	0.18	0	0	0	0	0	0.09	0	0	0	0	0.04	0	0	0
G02	44		0.00	0	0	0	0	0	0.17	0	0	0	0	0.01	0	0	0.03
G03	38	47		0	0	0.05	0.05	0	0.06	0	0	0	0	0.02	0	0	0.06
G04	52	53	48		0.05	0.02	0.14	0	0	0	0	0	0.14	0.03	0.13	0.09	0
G05	52	52	54	45		0.03	0	0.01	0	0.05	0.01	0.02	0.00	0	0.07	0	0
G06	51	53	45	48	49		0.12	0	0	0	0	0	0.06	0.06	0	0	0
G07	49	51	42	39	50	42		0	0	0	0	0	0.15	0.16	0	0.14	0.00
G08	53	54	48	52	48	53	52		0	0.17	0.09	0.09	0	0	0	0	0.05
G09	42	39	41	50	59	53	52	48		0.01	0	0.00	0	0	0.01	0	0.08
G10	50	49	50	49	44	53	50	38	43		0.33	0.37	0	0	0.01	0	0
G11	56	55	56	55	50	59	57	46	49	27		0.29	0	0	0	0	0
G12	55	54	55	54	49	58	55	45	48	24	34		0	0	0	0	0
G13	50	49	45	39	47	44	38	51	48	50	56	55		0.06	0.09	0.11	0
G14	46	50	45	46	52	47	39	55	50	53	59	58	43		0	0.01	0
G15	50	48	49	40	44	52	51	51	44	45	51	50	41	51		0.09	0
G16	50	50	46	41	50	53	39	48	46	46	53	51	40	46	41		0
G17	49	47	42	50	52	50	46	46	42	48	54	53	47	50	48	49	

Table 1. Matrix of individual Euclidean genetic distance (bottom) and kinship coefficient ϕ (top) between the 17 *Eucalyptus imlayensis* genotypes (3165 SNPs, 17 samples). Darker shaded genetic distance values indicate greatest differences, while darker shaded kinship coefficients indicate closer relationships between genotypes.

first-degree relationship between them. Paired samples collected as separate stems from the same lignotubers (*103, 103a; 115, 115a; 117, 117a*) belong to the same genotype as their pairs (*G2*, *G5*, *G7*, respectively). This confirms that stems from the same lignotuber and assessed as the same plant in the field were clonemates. However, some ramets were identified from apparently separate lignotubers so the extent of each clone cannot be determined visually.

Variation across the dataset and the genetic clustering of samples (genets and ramets) are depicted in the PCA (Figure 3), with axes 1 and 2 accounting for 29.62% and 18.45% of the variation in the dataset respectively. Clusters indicate clonemates whereas samples distanced away from clusters are unique genotypes of varying kinship. Two unique genotypes, *G11* and *G12*, had a first-degree relationship to genotype *G10* and to each other, indicating full sibling or parent-child (Table 1), with ϕ values ranging 0.29–0.37 and genetic distance values ranging 24–34. The neighbour-joining tree also presents visually the relationships between the colour-coded clonal groups and unique genotypes shown in black (*see* Suppl. file 4). The nesting of genotypes *G11* and *G12* within the group of samples assigned to *G10* in the neighbour-joining tree and in the PCA displays the close relationship between these genotypes. Eight genotype pairs had a second-degree (half-siblings, grandparent-grandchild), and all remaining pairs had a more distant relationship to any other genotype (Table 1) with ϕ values of 0 and genetic distance values generally >45 for distantly related pairs. The probability of Identity by Descent is shown for all 17 *E. imlayensis* genotypes and provides a visual overview of the relationships among genotypes that could be used for breeding selection (Figure 4). Genotypes that are closer to red have a higher probability of similarity, supporting the relationships shown in the neighbour-joining tree, such as the similarity between *G10*, *G11* and *G12*.

Discussion

Extensive clonality has been substantiated in *Eucalyptus imlayensis,* a rare mallee species, with only 17 unique genets identified for the species from the 48 tagged plants. This represents a sizable increase from the five genotypes identified in an earlier study where only part of the population was accessible (James & McDougall 2007). There was no evidence of seedling recruitment after the fire of 2019/20 suggesting a lack of soil-stored seed. The removal of dense vegetation by fire enabled comprehensive sampling across the entire site and the collection of reliable baseline data on the extent of the population and its genetic composition.

Measures of genetic diversity can be used as a proxy for a species' evolutionary potential, which is likely to be a decisive factor in determining its ability to adapt and persist (Sgrò *et al.* 2011). Over time, changing climate can create suboptimal environmental conditions and affect a species' resilience, including reduced fecundity with a shift in reproductive strategies (Silvertown

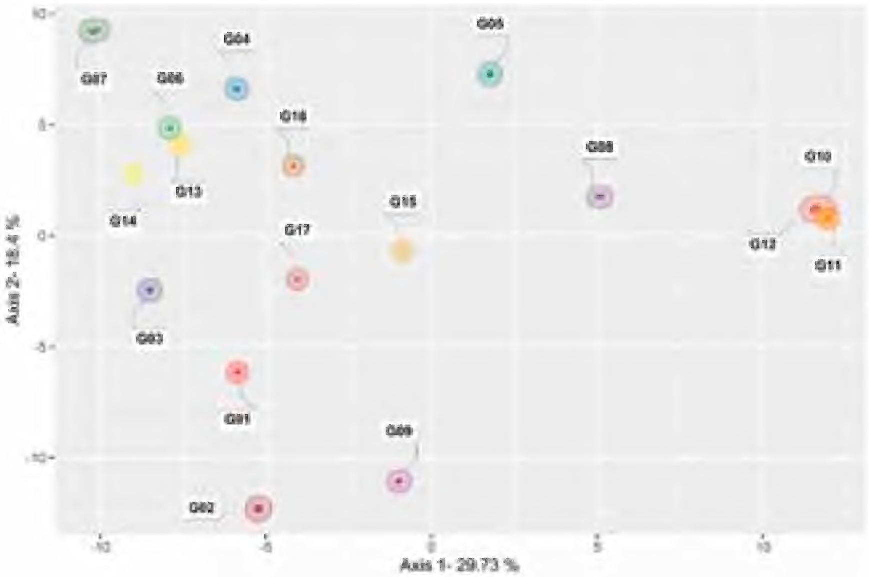

Figure 3. Principal components analysis of genetic differentiation for the set of 2980 SNPs for 50 *Eucalyptus imlayensis* individuals, colour-coded by genotype, with recurrent genotypes displayed with a circle and unique genotypes displayed with a triangle. See Suppl. file 2 for samples assigned to each genotype.

2008) and a tendency to succumb to environmental stressors resulting in the loss of intraspecific lineages (Levin 2019). Species restricted to a single population or few populations with few genotypes are consistent with remnant species rather than with neospecies, as suggested by Crisp and Brooker (1980) when describing *E. imlayensis*. A multi-species multi-population phylogenetic analysis to evaluate the relationship of *E. imlayensis* with both narrow-ranged and widespread relatives would provide context for developing conservation strategies within a wider biodiversity framework (Rossetto *et al.* 2021) but was beyond the scope of this study.

While many of the *Eucalyptus imlayensis* ramets

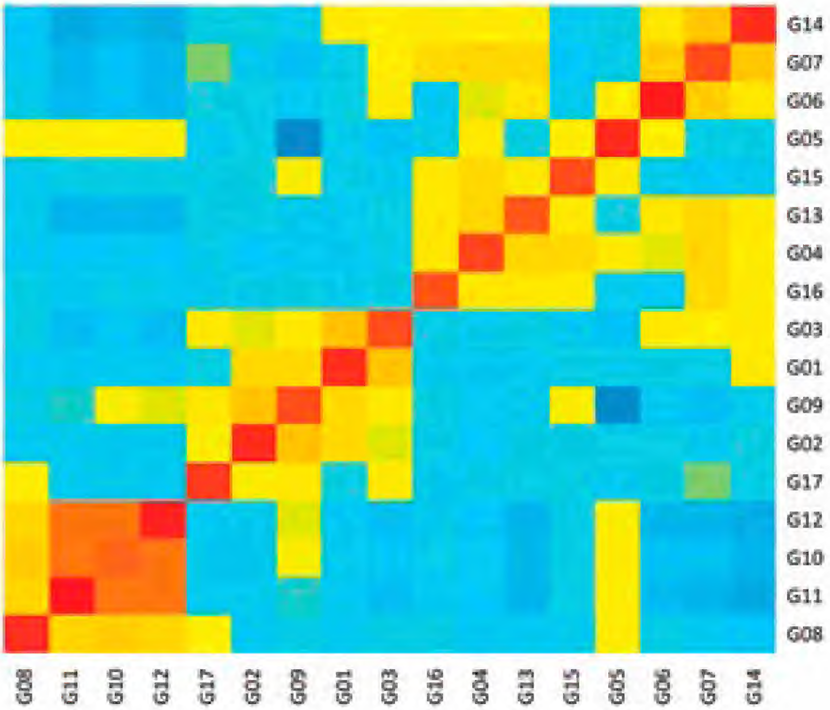

Figure 4. Heatmap showing probability of Identity by Descent for the 17 *E. imlayensis* genotypes (3165 SNPs, 17 samples). Each small square in the IBS distance matrix represents the genetic distance value between two pairs of genotypes. Higher probability values are shaded in red, indicating closer relationships between genotypes, while blue indicates lower probability and less closely related genotypes.

identified in this study corresponded to previously tagged clones, loss of a genet since 2007 is consistent with *E. imlayensis* being a relict species reliant on clonal growth and impacted by the loss of genotypic diversity over time. All sampled stems were resprouting from lignotubers and in some cases, stems from apparently separate lignotubers were found also to be clonemates. Clones varied in size with the largest (*G10*) extending over an area of 236 m^2. Size difference may be due to differences in the ages of clones but competitive advantage may also favour the expansion of some clones over others. Clone *G10* appears to have become fragmented because lignotubers with ramets belonging to clone *G10* were no longer obviously connected physically, or necessarily even the closest. Clones of woody species such as eucalypts are difficult to age because the lignotubers expand and fragment over time, losing obvious signs of previous physical connection. Several estimates of clone age in mallee eucalypts suggest that individual genets can survive for thousands of years (*E. curtisii*, Smith *et al.* 2003; *E. argutifolia*, Kennington & James 1997; *E. phylacis*, Rossetto *et al.* 1999; *E. absita,* Bradbury *et al.* 2016). Our findings are in keeping with what has been found in other rare, clonal eucalypts and clones of *E. imlayensis* are likely to be of a similar age.

Clonal growth confers some resistance to disturbances such as fire by enabling persistence when sexual reproduction is infrequent (Bond & Midgley 2003, Gross & Caddy 2006). The combined effects of drought, fire severity and frequency, and plant growth characteristics are likely to be elements in the persistence of fire-affected plants but their impact on the effectiveness of resprouting is understudied in Australian temperate forests (Bendall *et al.* 2022). Fire frequency is known to reduce the ability of eucalypts to resprout from lignotubers if the interval between fires is too short for energy reserves to be replenished (Fairman *et al.* 2019). Shorter fire intervals can be detrimental to seed production and seedling establishment leading to increased reliance on vegetative reproduction. Historically, fire occurred infrequently on Mt Imlay (McDougall *et al*. 2023), reflected in the high number of obligate seeders present. The lignotubers of *E. imlayensis* clearly provided a level of fire resilience to the 2019/2020 fires but greater mortality may occur if climate change driven increases in fire frequency and severity reduces a plant's ability to re-sprout (Fairman *et al.* 2019; Bendall et al 2022).

At present, very little seed of *Eucalyptus imlayensis* is stored in conservation seedbanks due to often unreliable natural seed production. To improve the conservation value of future seed collections, information about seed viability and paternity of open pollinated progeny arrays is still needed to determine whether viability and seedling vigour are affected by parentage. Separate storage of seed lots is vital for maintaining maternal lines and biparental crosses in conservation germplasm collections (Commander et al. 2018), and this is particularly true for mallee eucalypts, where seed production is low (Wellington & Noble 1985), as well as for many other predominantly clonal species (Bond & Midgley 2003). Factors contributing to reduced fecundity could include the differential effects of inbreeding and self-incompatibility found in some eucalypt species (Horsley & Johnson 2010; Nicklas *et al.* 2019) or an accumulation of somatic mutations that affect sexual but not vegetative reproduction (Gross *et al.* 2012). Lignotubers may also be a growth characteristic that enables species with low fecundity to persist where site conditions are rarely conducive to seedling establishment even when some viable seed is produced. The viability rates of seed from *E. imlayensis* is not known, but seedlings have been grown successfully *ex situ*. Natural seed production at the remnant site has been observed to be infrequent, low in number, and sporadic, and time to flowering and hence seed production in the population post-fire has been estimated to be at least 10 years (McDougall et al. 2023).

The small number of *E. imlayensis* genotypes means that it is feasible for all to be represented in *ex situ* conservation collections and these would provide a valuable genetic resource for breeding or seed production programs (Amor *et al*. 2020). *Ex situ* collections are ideal for maintaining control over genotype, creating insurance populations, and providing germplasm, including pollen, for experimentation and seed production in the field and off-site. However, eucalypts are often difficult to propagate vegetatively and previous attempts to graft *E. imlayensis* onto related species (e.g. *E. dalrympleana*) have not been successful (Peter Bredell, Australian National Botanic Gardens, pers. comm. 2022). Micropropagation methods that have been successful for the multiplication of eucalypt germplasm (Trueman *et al.* 2018) may provide a better option, although the lead time for micropropagated plants to flower is unknown.

Despite the presence of 17 genotypes, many individuals are closely related which could affect the success of a breeding program and there are still many biological unknowns about the species. For sexually reproducing species, erosion of genetic diversity is a risk when populations are limited. Where sexual reproduction is compromised there may be little scope for genetic recombination or to maintain existing genetic diversity. The historical loss of intraspecific lineages of *E. imlayensis* resulting in closely related extant individuals may be a factor in a compromised mating system. Identification of *E. imlayensis* genotypes and measures of their relatedness provides a genetic basis for examining breeding and pollination systems. Studies of widespread eucalypts have shown a bias towards outcrossed seed or germinants and a deficit of homozygotes. This is thought to be due to the purging of individuals resulting from self-fertilisation in open pollinations containing mixed pollen loads (Horsley & Johnson 2010, Nickolas *et al.* 2019). Little is known of the seed viability in *E. imlayensis*, and whether selection against homozygosity post seed production occurs as it does for other rare clonal mallees (Kennington & James 1997).

Genetic interventions may be warranted for conservation targets using genetically optimised population augmentation or introduction (Sampson & Byrne 2016), manipulated pollinations to minimise genetic erosion (Cook & Sgrò 2019) or translocations from *ex situ* collections (Thomas *et al.* 2021; Rutherford *et al.* 2022). A workflow, adaptable for other species, was designed by Bragg *et al.* (2021) to ensure that *ex situ* collections of an endangered rare eucalypt, *Eucalyptus* sp. Cattai, were genetically optimised for conservation by manipulating the genetic diversity represented. With additional research, keeping in mind that the available gene pool is highly restricted, a pollination program could be implemented for *E. imlayensis* that minimises inbreeding while maximising the retention of genetic diversity. Self-fertilisation and biparental inbreeding should be avoided. Seed production from crosses between the most genetically distinct lineages of *E. imlayensis* (pairs of genotypes represented by lighter colours in Table 1) should minimise the loss of diversity and reduce the risks associated with inbreeding. New plants grown from seed, each of a unique genotype, have the potential to contribute to improving genetic resilience due to recombination during sexual reproduction. Even so, with so few extant *E. imlayensis* genotypes, it may be necessary to perform controlled pollinations between any genotypes of plants flowering concurrently rather than the ideal of plants of greatest genetic distance.

The conservation of a species restricted to a single, small population provides both opportunities and novel management challenges, including a restricted gene pool. Competition and site conditions are likely to limit seedling establishment and options for augmentation or translocation. While the remnant site is not considered suitable for population enhancement due to its to rocky profile and shallow soil, 40 tubestock seedlings grown *ex situ* were successfully established near the remnant site on Mt Imlay in 2011 and 2018 (McDougall *et al.* 2023). Thirty-seven of these plants survived until they were killed in the 2019/20 fires. This outcome highlights the vulnerability of plants that have not yet developed sufficient protection for buds or the ability to resprout after high intensity fire. However, the assisted establishment of seedlings suggests that additional translocation enhancements may be a feasible conservation option if sufficient seed is available, and the chance of survival is deemed to outweigh the risk of fire impacting the site before plants have established lignotubers.

The future persistence of *E. imlayensis* is likely to be affected by various factors, not all of which can be controlled, including ongoing climate change, increasing fire frequency and intensity, competition from other species such as *Leptospermum scoparium*, and potentially the impact of disease-causing agents such as *Phytophthora cinnamomi* (Department of Environment and Energy 2018). The practicalities of establishing a genetically representative *ex situ* population, success of cross-pollinations and seed production, feasibility of storing viable pollen and the cost effectiveness of their implementation are still to be evaluated by conservation managers, but the high priority conservation actions for *E. imlayensis* are those that are most likely to be achievable and to contribute to the species future persistence. They include a genetic focus on collecting seed from the wild, testing the genetics and viability of seedlots, and expansion of the genetic representation and number of stored seeds from both natural and controlled pollinations. Additionally, an *ex situ* collection is not only valuable as insurance against repeated fires but will provide an experimental laboratory for undertaking breeding system studies and support on-ground application of a genetically supported conservation program for *E. imlayensis*.

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Supplementary files

1. Unfiltered matrix genetic distance, unfiltered, all samples (55, including technical replicates)
2. Matrix of kinship values filtered (50 samples, no technical replicates)
3. Heterozygosity and fixation indices for 17 individuals (one representative of each of 17 genotypes)
4. Neighbour-joining tree showing clusters of *Eucalyptus imlayensis* stems based on genetic similarity (50 dataset). Samples eim131 and eim142 (G11 and G12, respectively) were identified as first-degree relatives of G10 clonemates and are embedded with them. Samples eim107, eim120, eim125, eim140 and, eim148 are unique genotypes G13, G14, G15, G16 and G17, respectively.

Supplementary files 1–4 are appended to the online listing of this paper, accessible via: https://www.rbg.vic.gov.au/science/journal/

Muelleria

41: 29–33

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ROYAL BOTANIC
GARDENS VICTORIA

Confirmation of *Olearia aglossa* (Asteraceae) in Victoria with notes on its distribution

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Abstract

Previously *Olearia aglossa* (Maiden & Betche) Lander has cautiously been treated as present in Victoria. This was due to confusion around diagnostic characters, similarity with other more widespread species, and uncertainty around its relationship with *O. alpicola* var. *rhodochaeta* F.Muell. Following inspection of type material and study of wild growing plants, these two taxa are shown to be synonymous. *Olearia aglossa* is here confirmed as present in Victoria, and details of its distribution and the features that distinguish it from morphologically similar species *O. alpicola* (F.Muell.) F.Muell. ex Benth. and *O. megalophylla* F.Muell. ex Benth. are discussed.

Keywords: Cobberas, *Olearia alpicola*, *Olearia megalophylla*, rediscovery

Introduction

Olearia aglossa has long been a poorly understood species, which has likely been often overlooked due to its close affinity with *O. alpicola* and *O. megalophylla*. Maiden & Betche (1899) described this taxon (as *Olearia alpicola* var. *aglossa* Maiden & Betche) as being distinguished from *O. alpicola* by the 'absence of the ray-flowers, in the fewer flowers in the heads (5–7 in the heads examined) and in the pinkish tint of the pappus bristles'. They noted that Mueller's material from the Cobberas – described by Mueller (1860) as *Eurybia alpicola* var. *rhodochaeta* – 'agrees precisely' with this species except for the presence of ray-flowers. Despite noting the affinity, they do not appear to include Mueller's specimen in var. *aglossa*. Lander (1991) elevated *O. alpicola* var. *aglossa* to species rank and expanded the description to include plants with up to 4 ligules per capitulum. Lander (1991) listed var. *rhodochaeta* as a possible synonym as he had not seen any of Mueller's specimens to confirm the association of the two taxa. The provision for *O. aglossa* to also include plants with ligules suggests they are the same taxon, as this was previously thought to be the only character distinguishing the two taxa.

Lander's (1991) change potentially expanded the distribution of *Olearia aglossa* to include Victoria, with Mueller's type of var. *rhodochaeta* the only record in Victoria. Walsh and Lander (1999) cautiously included this species in the *Flora of Victoria*, noting that its presence in Victoria relied on the taxonomic concepts of Mueller and Maiden & Betche being conspecific. The existence of *O. aglossa* in Victoria has up to this

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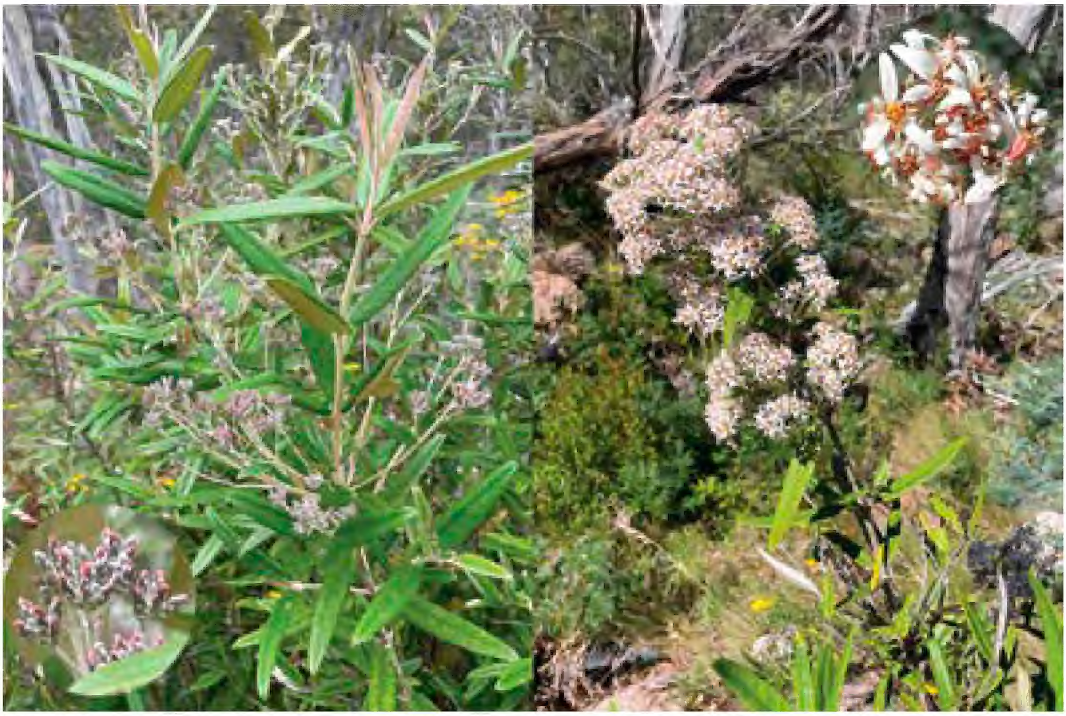


Figure 1. Mount Stradbroke population of *Olearia aglossa* in bud (left) and full bloom (right), inserts showing close up of capitula.

point remained unclear, with no other unambiguous specimens of this taxon held at MEL, and at least 140 years since its last sighting within the state.

Recent findings

In early 2023, plants matching *Olearia aglossa* were discovered on Mount Stradbroke, eastern Victoria, only 53 km south of the type location (Mount Kosciuszko) and 15 km southeast of the Cobberas (type location for var. *rhodochaeta*). The population was found in early February, and at that time all plants appeared to have highly reduced ray-florets. The discovery led to the conclusion that *O. aglossa s.s.* had at last been confirmed in Victoria. During a subsequent visit in March 2023, plants were observed in full bloom and had developed fully expanded ligules to 10 mm long (Fig 1). This prompted an investigation of the type specimens of *O. aglossa* for comparison. During this work, it became apparent that a type specimen for *O. alpicola* var. *rhodochaeta* has since been located at MEL – subsequent to the *Flora of Victoria* treatment. Inspection of this specimen, along with types of var. *aglossa*, and comparison with field observations, confirmed that they are conspecific. The only character that differs between type specimens of these two taxa is the absence of ligules on Maiden's syntype specimens. However, close inspection of these specimens revealed they are in bud, with neither ray or disc florets open. The reason ligules were not observed is because they have not yet developed and expanded. All three type specimens cited by Maiden & Betche (1899) were collected in January and are undoubtedly at the same immature stage as plants observed in early

Key to species

1 Indumentum on lower leaf and peduncles of ascending Y-shaped hairs, making surfaces appear somewhat tangled and scruffy; leaves prominently reticulate-veined above; involucre cup-shaped, innermost bracts usually more than 6 mm long.......... ***O. megalophylla***

1: Indumentum on lower leaf and peduncles of appressed T-shaped hairs, appearing somewhat smooth or silky; upper leaf surface not or indistinctly reticulate-veined; involucre conical, innermost bracts less than 6 mm long 2

2 Ray florets 2–4; pappus bristles often pink; leaf length:width ratio usually <6 .... ***O. aglossa***

2: Ray florets 5–8; pappus bristles white to straw-coloured, never pink; leaf length:width ratio usually >6.......... ***O. alpicola***

February 2023 at Mount Stradbroke. Plants collected in full bloom at Mount Stradbroke in March 2023 match the type of var. *rhodochaeta* (Fig. 1).

It is unfortunate that Lander (1991) had not seen the type specimen of var. *rhodochaeta* and was hence unable to synonymise the two. Lander's (1991) description allowed for the development of ligules, therefore the name *aglossa* (without tongues – alluding to the lack of ligules) is a misnomer. It is now clear that the type specimens of var. *aglossa* would have displayed developed ligules had they been collected a few weeks later. Mueller's epithet *rhodochaeta* (rose-bristles – alluding to the distinctive colour of the pappus) while also not diagnostic, appears to be a much more fitting name. Despite *rhodochaeta* being the earlier name, the priority principle does not apply in this case as the epithet is now being used at a different rank (see ICBN Article 11.2). Attempts to change this name here are likely to result in unnecessary additional nomenclature and further confusion. Furthermore, a new combination is likely to be made for this species in the future (along with most Australian taxa in section *Dicherotriche*) as phylogenetic studies have shown them to be unrelated to *Olearia s.s.* (Cross et al., 2002). Any nomenclatural changes made here would be short-lived.

Distribution of *Olearia aglossa*

The uncertainty (at least in Victoria) around the reduction of ligules – taken literally as a reduction in size and number, rather than just number of florets as a key character, has led to uncertainty in the identification of *Olearia aglossa* specimens at MEL. With this character now clarified, a number of other specimens at MEL have been attributed to *O. aglossa* providing a much better understanding of the distribution of this species in Victoria. *Olearia aglossa* has been recorded from scattered sites in highlands in the far east of Victoria (Fig. 2), with specimens from the Cobberas, Mount Stradbroke, Gelantipy, Snowy River area, Mount Tingaringy, and Mount Koolabbra (between Bendoc and Bonang). At nearly all sites plants occur in sclerophyll forest in dry, rocky situations.

In New South Wales, *Olearia aglossa* is currently known only from Mount Kosciuszko National Park and an isolated record further east at 'Ngarago', 24.5 km ESE of Nimmitabel. Despite Lander's revision and the less ambiguous key provided in the *Flora of New South Wales* treatment (Lander 1994), there are only seven specimens recorded of this species in New South Wales (three of which are the syntypes). A revision of specimens stored under *O. megalophylla* and *O. alpicola* held at NSW is likely to uncover additional locations of *O. aglossa*.

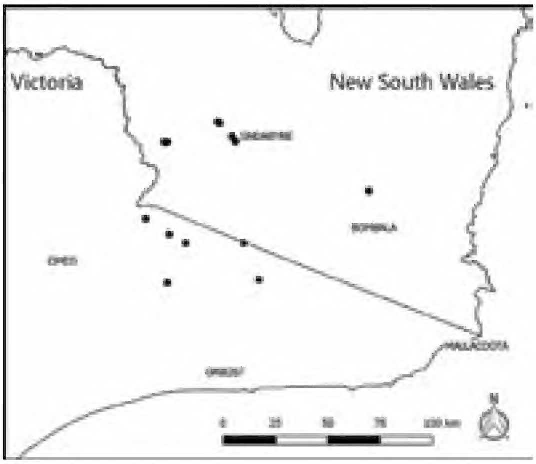


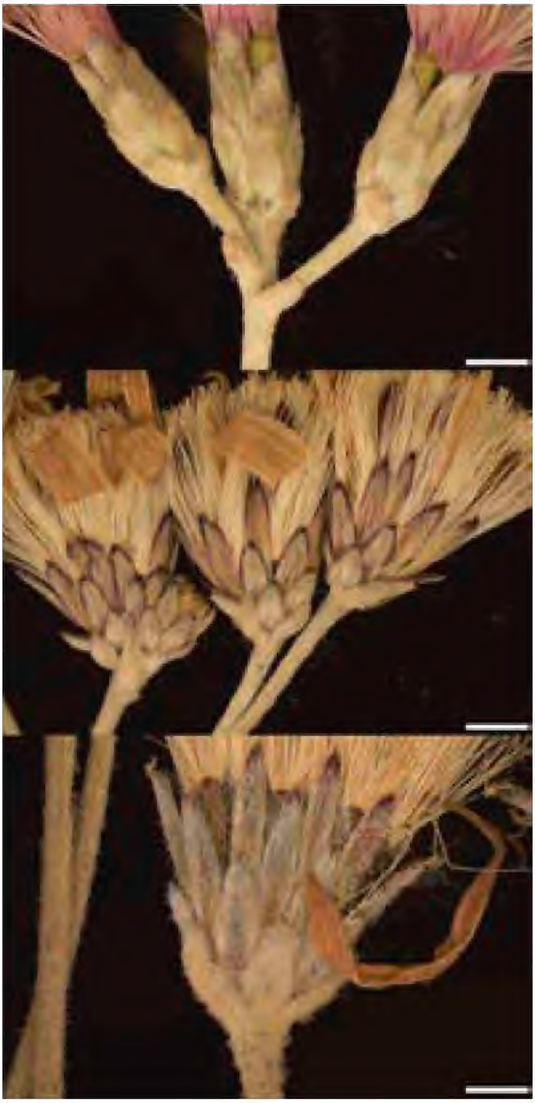
Figure 2. Distribtion of *Olearia aglossa*. Data provided by The Australasian Virtual Herbarium, see https://avh.chah.org.au/

Diagnostic features

Olearia aglossa has been confused with *O. alpicola* and *O. megalophylla* – two species which may also at times be difficult to distinguish. A table of diagnostic features and an identification key for these three species is provided here. In terms of leaf shape *O. aglossa* can be quite similar to *O. megalophylla*, with both species having relatively broad leaves that differ in overall shape and width to those of *O. alpicola* (Table 1). The indumentum of the stems, lower leaf surface, and bracts of *O. aglossa* are similar to those of *O. alpicola*, both having appressed T-shaped hairs (Fig. 3, Table 1), giving surfaces a smooth or silky appearance (*c.f.* ascending Y-shaped hairs, making surfaces appear somewhat tangled and scruffy in *O. megalophylla*). *Olearia aglossa* and *O. alpicola* are also similar in involucre size (Fig. 3, Table 1), both being narrower, with shorter bracts in more numerous rows than *O. megalophylla*. However, *O. aglossa* can be further distinguished from both of these species by the reduced number of florets, with heads containing only 2–4 radiate florets and 2–4 disc florets (*c.f.* 5–9 ray and >9 disc florets in both *O. aglossa* and *O. megalophylla*). *Olearia aglosssa* has also previously been characterised

Table 1. Morphological comparison of *Olearia aglossa*, *O. alpicola* and *O. megalophylla*.

		O. aglossa	***O. alpicola***	***O. megalophylla***
Leaf	Shape	Lanceolate to narrow-ovate	Narrow linear, oblong, narrow-ovate	Ovate, lanceolate, narrow-elliptic
	Length (mm)	(40–)55–90(–115)	60–130(–140)	(25–)55–130
	Width (mm)	(10–)13–27(–35)	3–12(–17)	(10–)13–35(–40)
	Venation above	Indistinctly reticulate	Indistinctly reticulate	Strongly reticulate
Hair	Type on leaf lower surface, stem and bract	Appressed T-shaped	Appressed T-shaped	Ascending Y-shaped
Capitulum	Involucre shape	Narrowly conical	Conical	Cup-shaped
	No. rows of bracts	4–6	4–6	3–4
	Inner bract length (mm)	4.5–5.5	4.5–5.5	(5–)6–9
	No. ray florets	2–4	5–8	5–9
	Ligule length (mm)	4.5–10	5–10	8–14
	No. disc florets	2–4	9–11	9–14



LEFT: Figure 3. Comparative images of involucral size and hair types. *Olearia aglossa* (top), fresh material (*A.Messina 2508*, MEL2491335); *O. alpicola* (middle), dried specimen (*A.Messina 2425*, MEL2527988); *O. megalophylla* (bottom), dried specimen (*A.Messina 2473*, MEL2528036). Scale bar represents 2 mm.

by the bright pink pappus bristles. However, pappus colour was found to vary within the population at Mount Stradbroke from very bright pink to pale pink, straw-coloured with a pink tinge, to straw-coloured. Pink pappus has apparently never been observed in *O. alpicola*, but is sometimes seen in other species in *Olearia* sect. *Dicerotriche*, including *O. megalophylla* and *O. myrsinoides* (Labill.) Benth. This feature, while at times distinctive, should not be treated as diagnostic.

Concluding remarks

It is hoped that this will paper will raise the profile of this species, which has been overlooked due to its resemblance to the far more widespread *Olearia alpicola* and *O. megalophylla*, both of which occur in close proximity to known *O. aglossa* populations. The characters provided in the key to the species are sufficient for straightforward identification. This is not intended to be a taxonomic treatment of these species, as this is provided in Lander (1991) while an updated treatment can be found on VicFlora (2023).

Acknowledgements

Plants of *Olearia aglossa* were found during a BushBlitz biodiversity discovery expedition to the Australian Alps. The author would like to thank

Laura Simmons for generating the distribution map, Renee Gugiatti (PERTH) for providing a specimen image, and Tom May for assistance with nomenclature. Finally, Neville Walsh is thanked for always reminding the author to look out for this "weird, pink-coloured *Olearia*" during all visits previously made to the Cobberas region.

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Muelleria

41: 34–43

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ROYAL BOTANIC
GARDENS VICTORIA

A new edition of the 'Published works of Ferdinand J. H. Mueller': bibliographical issues and questions of effective publication of ostensible preprints

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Abstract

A new edition of the list of Ferdinand (von) Mueller's published works has been prepared, based upon that originally published in *Muelleria* in 1978 and 1984, and then later revised in 1998. The new edition is one of several supporting documents prepared by the editors of the von Mueller Correspondence Project (https://vmcp.rbg.vic.gov.au/). The bibliographic implications of multiple contemporary republications of Mueller's articles and his use of extra-prints are discussed. Mueller's widespread use of extra-prints is examined and factors to be considered in determining effective publication status are discussed and exemplified.

Keywords: bibliography, reprints, extra-prints

Introduction

A new edition of Mueller's publication list (https://vmcp.rbg.vic.gov.au/text/static/apparatus/muellers-publications/) has been published 45 years after the first edition (Churchill et. al. 1978). Since then, access to literature sources has become much easier, including especially to non-scientific literature such as newspapers that are of major importance in understanding the reach of Mueller's views and interests. Digitised page images make it possible to produce a bibliography without having to be based in a library and using inter-library loans. Specialised aggregators such as the Biodiversity Heritage Library (https://www.biodiversitylibrary.org/) are well known, as is the non-specialist Internet Archive (https://archive.org/), but tools that allow searching of traditional library catalogues, especially those that give links to digital versions of holdings, are also important. The *Karlsruhe Virtual Catalog* (https://kvk.bibliothek.kit.edu/) is a generally useful international meta-catalogue of this type. For newspapers, the digitisation project of the Australian National Library (https://trove.nla.gov.au/newspaper/) and equivalents for other countries are invaluable, provided one uses a variety of search terms to compensate for un-proofed text generated by Optical Character Recognition. 'Miiller', 'Muellor', 'Muollor' and similar variants were routinely used as search terms, but no such variant would have picked up items where the name was rendered 'Hneuer' or 'Slvtoil'sr', noticed when searching for 'government botanist', or 'Baron von'.

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I remain impressed, however, by the excellent coverage of the botanical literature achieved by the 1978 editors (Maroske (2018) discusses Sinkora's role). Although there are some new technical items, most of the additions are from newspapers in Australia, New Zealand, the United Kingdom and the United Sates, mainly California. They illustrate the importance Mueller gave to applied botany, with many items on poisonous plants and suggested crops, and to exploration. Newspapers were also tools to help him recruit potential collectors via advertisements and letters to editors.

Preparation of the edition of Mueller's correspondence and the discovery, facilitated by digitisation projects, of reviews of his works in journals and newspapers, has enabled more accurate publication dates to be determined for many items. To allow easy access to the new information in situations where the older numbers have been cited in publications, this new edition follows the pattern established in Home *et al.* (1998) to cross-reference old bibliography numbers for the approximately 350 items that have been re-dated, including around 40 that were re-dated in 1998. Similarly, items re-published in full or in substantial part have been mutually cross-referenced by using a 'see also' note. Almost 850 of the around 2,800 substantive entries are additional publications, leaving a still impressive number of around 2,000 titles, almost double the number in the first edition in 1978.

A new section has been added to this updated edition to list spurious attributions of authorship, many from contemporary sources, especially Knapp (1877) and *Repertorium annuum*. These are not discussed further here, as the section 'Spurious attributions' in the new edition included with the *Correspondence* (Home *et al.* 2023+, Apparatus, Mueller's publications) includes full details.

Bibliographic issues

Several issues arose when the editors were revising Mueller's bibliography, more properly considered as a 'publication list' since it excludes literature about him. What to include seems to be obvious – anything published that Mueller wrote should be listed. However, that is a difficult demarcation criterion. This of course is not a problem unique to Mueller:

> Readers of bibliographies are typically so thankful for what they find that they are all too quick to forgive for what they miss, leaving it incumbent on the compiler to work out with God the particulars on what really belongs there: how much of the decision deserves to be explained in the introduction (and God has probably been rather too forgiving in this matter), and how much can be accommodated under the simple pragmatic rule of "err on the side of inclusion." (Krummel 1988, p. 244).

In Mueller's case several different kinds of demarcation problem arose where judgement was necessary.

Should, for example, interviews and reports of speeches qualify? The bibliography includes several speeches, and one interview that appears in five versions (see item 79.09.04).[1] These were included as they are valuable evidence of Mueller's opinions and activities, especially outside botany and applied botany. But to be included they needed to be, as far as could be judged, close to verbatim records: summary reports of speeches were excluded.

What about advertisements? Some Mueller placed himself, asking for specimens; others are quotations from his publications, used by merchants extolling their wares. The first category is clearly relevant, being explicitly written for publication: see, for example, 48.09.01, where Mueller sought subscribers to exsiccatae he intended to prepare, and 60.01.03, the call for live specimens of 'the AUSTRALIAN MAGPIE and LAUGHING JACKASS' to be sent to Britain by the Acclimatisation Society of Victoria. The latter appeared almost daily in the same newspaper for nearly a year, but the repeat insertions are not individually listed, with a note to that effect in the entry. The second category is less clear-cut, but two instances are included, 69.04.02 in a Western Australian newspaper, that quotes from 69.01.03 to support the claims of a manufacturer of 'patent chemical manures', and 80.08.04, an extract from 76.12.04, used in an advertisement of a Sydney seed merchant in the *Los Angeles Herald*. They are, in essence, equivalent to other partial republications, issues with which are discussed below.

Mueller was an active translator, both of articles – for example 63.10.05 in a farmers' journal, and 69.01.01 in the *Australian Medical Journal* – and of one phytochemical book, 78.06.09, which he expanded. He was often translated, for example versions of his essay on the vegetation of Victoria (61.10.02) appeared in German (61.13.09) and French (61.13.10). All known contemporary translations are listed, and notes but not individual listings made of modern translations – for example the translation by Darragh of 50.11.01 (supplementary material to Maroske & Darragh 2016) – although an exhaustive search has not been made for similar items.

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[1] When pointing to exemplifications of criteria, citations to Mueller's publications use the number given to the item in the file in https://vmcp.rbg.vic.gov.au; elsewhere the entry number is given as well as the standard citation.

Letters 'to the Editor' are unproblematic, as intended for publication. Similarly, letters to third parties that were then forwarded for publication are included automatically, whether they were supplied by a Government official, for example 75.05.04 on the poisonous properties of feed containing darnel seed, or by a private individual, for example, a letter, 92.07.07, sent to E. Leary on the terminology to be used in describing the natural order to which nightshades belonged that Leary published as part of his public argument with another newspaper correspondent. Published quotations from letters, on the other hand, require judgement. It would be absurd to include as a Mueller publication the quotation 'Is there any state of the Ficus australis in Illawarra or elsewhere in New South Wales producing a Banyan-like growth such as occurs in the Ficus columnaris of Howe's Island?' attributed to him in the introduction to Bennett (1877). However, a quotation from a letter sent to O. W. Sonder used in a note by Sonder to *Hamburger Garten- und Blumenzeitung* (58.04.02) must qualify, as to exclude it would be to hide important details of what European botanists learnt of Mueller's activities. Similarly, although short, comments solicited by agricultural and horticultural editors of the weekly newspapers on behalf of their correspondents are included when quoted verbatim, for example, 85.02.06, 94.10.03. To omit them would remove evidence of Mueller's very important, very visible, role in public service.

Reprints and republications

Republications of whole articles are automatically included. Some, such as those in German periodicals of articles that Mueller published in the *Victorian Naturalist,* were undoubtedly forwarded by him (compare 89.04.04 with the source 89.03.02). It is less clear whether Mueller or the editors of the republishing journal translated those that were printed in German; for example, the editors of *Hamburger Garten- und Blumenzeitung*, who added a commentary to 85.01.04, may also have translated it from the source in 84.10.03.

Judgement is required, however, when a republication is an extract of another item. Some are unproblematic, for example the serial republication of Mueller's *Introduction to botanic teachings…* (77.08.01) in California as 77.11.04 and linked items and in New South Wales as 78.01.02 and following articles. (For the circumstances of the NSW republication see Lucas *et al.* 2006, section 3.3, especially pages 42–43.) By contrast, *Suggestions on the maintenance, creation and enrichment of forests* (79.05.06) was widely reviewed, often with short quotations, and was summarised in 1883 in *Just's Botanischer Jahresbericht* **8**, 392-394. These short extracts and summaries are not given their own entries, although very substantial extracts from the work are listed, for example 79.07.05, which embeds the extracts in an editorial on forest policy for Western Australia.

Especially problematic are extracts from the various editions of *Select extra-tropical plants* (editions cross referenced under 76.12.04). These titles comprise entries under species names, with notes on economic value, geographic origin, culture notes and the like. Where the source is acknowledged and complete entries are given, these have been included when they are items in their own right but usually excluded when they are very short and used as part of another article. Most problematic is where they seem to be a *de novo* contribution, which might seriously mislead readers into thinking that the work described had been recently conducted. For example, in the *Colac Herald,* 29 November 1887, p. 3, the following comment was printed in a discussion of cocksfoot grass:

> no other (European) grass can be compared to it for copiousness of yield, particularly if the soil contains a fair quantity of lime; its nutrition being first class. The chemical analysis made very late in spring by Mueller and Rummell gave the following results:-Albumen, 1.87; gluten, 7.11; starch, 1.05; gum, 4.47; sugar, 3.19 per cent.

This looks like a new piece of information, but the data are taken from the entry 'Dactylis glomerata' in either Mueller (1881), p. 101 (entry 81.01.04), or the next Australian edition (Mueller 1885, p.115, entry 85.12.03). Such items are not included in the publication list, and if found by readers cannot be taken at face value but need to be critically examined for their provenance. Anything attributed to Mueller in newspapers relating to plants of economic value after the publication of the first version, *Select plants* (*exclusive of timber trees*) (Mueller 1872, entry 72.07.01) should be compared with his final edition of *Select extra-tropical plants* (Mueller 1895, entry 95.08.04), and if dating of the comment is critical, traced back through earlier editions.

Modern 'print on demand' versions, widely advertised on bookseller websites, and of problematic legitimacy as often taken from on-line images on digitisation sites, have not been included. Nor have legitimate non-contemporary reprints of some works been listed; for example, the 1974 facsimile reissue of the complete *Fragmenta phytographiae Australiae* by Asher has been omitted. (It contains the first privately printed fascicle, 58.03.01, not the reprinted version issued with 58.06.01 – see Darragh & Lucas, (2015)).

Proofs and extra-prints

> The specific epithet in the original publication is Tatea subacaulis whereas in this leaflet it is "Tatea acaulis". This has misled many botanist (*sic*). A.A. Munir. 29.xi.1983. (Final part of a note attached to K1067682)

Munir's note, drawing attention to two versions of a text by Ferdinand von Mueller, exemplifies the bibliographical problems posed by proof sheets and extra-prints. How should proof sheets be handled when they are known to have been sent to correspondents by Mueller? How should intended publications known by the existence of copies of proofs or extra-prints be treated if they were never formally published, or if they were published elsewhere, explicitly based upon texts received from Mueller? These questions are relevant not just for bibliographers but also for nomenclaturists, because considerations of 'effective publication' under the *International Code of Nomenclature for algae, fungi, and plants* (Turland et al. 2018) (previously the *International Code of Botanical Nomenclature*; hereafter 'the *Code*') arise from these cases.

Extra-prints of published articles. We know that Mueller was anxious to see his work in print quickly, and to receive printed copies as soon as possible. For example, he asked Otto Tepper in a letter of 20 February 1882 'How often does the Adelaide Royal Society publish and does the author receive his extra reprints right after the printing of the paper?'[2] He asked a similar question about being able to receive extra-prints at his expense if a paper was submitted to the Linnean Society of New South Wales.[3] Especially, but not only, after his *Fragmenta* ceased publication with 82.12.03,[4] it is very likely that the great majority of his papers published in journals were distributed as extra-prints, not just the few that were so listed in the 1998 edition of his bibliography. For example, we know that Tepper received a prepublication copy of the extra-print of 96.10.01 with a letter from Mueller written in the margin, for after Mueller's death in October 1896, Tepper so annotated a copy that Mueller had sent on 18 September.[5]

The majority of known distributions of extra-prints bear neither a date of despatch nor one of receipt, but the example of the sheet received by Tepper indicates that at least some of them were distributed before the item was formally published, in this case in an issue dated 1 October. The five volumes of Mueller's 'Opuscula' at Kew contain many items, some annotated as presentation copies without dates to Joseph or William Hooker or to George Bentham, as well as some that were originally presented to others who were not based at Kew. For example, there is a copy of 60.04.07, the *Report on the resources of the Colony of Victoria* (Royal Society of Victoria, 1860) inscribed by Mueller 'To his Excellency Sir Geo: Gray, K.C.B.'. The National Library of Australia holds a substantial number of reprints or proofs, mainly in the series of 'pamphlets' collected by each of the bibliographers E.A. Petherick and J.A. Ferguson, as well as by the collector Rex Nan Kivell. The majority of these are not listed in the Home *et al.* (1998) edition of Mueller's publications. Many are inscribed by

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[2] State Herbarium of South Australia, Plant Biodiversity Centre; Mueller Correspondence project translation. Mueller's question is a little odd, as earlier that year 82.01.02 had been published in the Society's journal. All letters cited here are included in the Mueller Correspondence Project collection; the archival location of the original is given in the notes.

[3] Ferdinand von Muller to Edward Ramsay, 22 March 1880, (ML MSS.562, Letters to E. P. Ramsay 1862–91, Mitchell Library, State Library of New South Wales).

[4] According to Mueller, the majority of the edition of this part was destroyed in the 1882 fire in the Government Printing Office, and although Mueller hoped to continue the series, and into it to 'collect what became lately scattered', no more parts were issued (Mueller to Robert Brain, 22 May 1889, M89/6614, unit 501, VPRS 3992/P inward registered correspondence, VA 475 Chief Secretary's Department, Public Record Office, Victoria; Mueller to George Bentham, 8 November 1882, RBG Kew, Kew correspondence, Australia, Mueller, 1882–90, ff. 38–39).

[5] Ferdinand von Mueller to Otto Tepper, 18 September 1896, Tepper's MS copy at RB MSS M198, Library, Royal Botanic Gardens Victoria.

Mueller, but they very rarely have a date of presentation. Petherick evidently acquired, presumably in London, many items once owned by the former Governor of Victoria, Henry Barkly, to whom Mueller continued to send reprints, the latest being a copy of 96.14.02, his joint paper with Tate.[6] Some, such as the leaflet noted by Munir in the Kew herbarium, are undoubtedly proofs, not extra-prints of the final article.

The general lack of distribution dates posed a problem for compiling the current edition of Mueller's publications. Should an effort be made to include all items for which a proof or extra-print is known to exist? If not, how should the very small number of such items listed in the earlier editions be dealt with?

It would be misleading to admit automatically any newly found extra-prints. Doing so would inflate unrealistically the number of Mueller's publications, and without distribution dates would not add value. We also 'deleted' the existing items, unless there was very strong evidence that it would be misleading to do so. 'Deletion' decisions are noted against the original bibliography number to allow comparability between editions, but a comment has been added: 'No explicit evidence has been found that proofs or extra-prints were distributed significantly before [bibliography number] was published'. If there is a strong possibility that copies were provided when the item was read to a Society but not issued as part of its *Transactions* or *Proceedings* until much later, they were not deleted but an annotation made. For example, 83.13.08, which was read at the meeting of the Royal Society of New South Wales on 5 December 1883 must have been distributed as an offprint before the volume of the Society's transactions in which it appeared was issued in August 1884: it was effectively republished in *Botanisches Centralblatt* in June (84.06.04). Similarly, the comment in the entry for 91.08.03 reflects the judgement that it is almost certainly a proof of Mueller's paper read to the Royal Society of Tasmania on 17 August 1891, a copy of which was sent to *Botanisches Centralblatt* in time for it to be published on 7 October 1891 (91.10.03), well before it was published in the Society's journal in June 1892 (92.06.02).

Warning about effective publication of items. Some of the items listed in the 1978 and 1998 editions of Mueller's publications are citations of extra-prints or 'galley proofs' of items also listed as an item in the relevant issue of the journal. A few of these items have a note stating that 'Further investigation may be required to determine whether the names were effectively published according to Article 29 of the International Code of Botanical Nomenclature (1972).'

Most of the problematic items refer to copies of versions of items that were not published as intended or expected. This is especially true of eight items destined for Wing's *Southern science record* in issues that never appeared. The earlier editions of Mueller's bibliography included the warning but gave no evidence that the items had been distributed, merely asserted that they had been. The working folders at MEL used to prepare these editions are of little help, only occasionally giving evidence of provenance of the copy held. It is, in principle, possible that the copies were received from the publishers by Mueller but not distributed in a way that would meet the criteria for effective publication. The editors therefore decided that where evidence of the existence of a copy other than at MEL was available it should be given in the note to the entry. Two examples suffice:

> Firstly, item number 84.13.18, where the entry is:
> [Extra-print] Definitions of some new Australian plants. *Southern Science Record*, **3**: 281-282. [This issue of the journal was never published. However, the article was distributed as an 'extra-print' by December when it was reported as being received by the *West Australian* (Perth), 30 December 1884, p. 3. It is summarised in *Just's botanischer Jahresbericht* **12**, Abt. 2: 204. There is a copy in the Australian National Library 'Botany leaflets', PETHpam 538, with a printed heading 'From Wing's "Southern Science Record," Vol. iii, pp. 281, 282.' A full Latin description of *Dipteranthemum* and the species *D. crosslandii* is given by Daniel Oliver in *Icones plantarum*, **16**, (1886), t. 1541, citing as the author of the name '*F. von Mueller in Wing's Southern Science Record*, iii, p. 281' A copy of the extra-print is attached to specimen K357034.]

In this case, only *Dipteranthemum crosslandii* of the three new species described was published in a journal, albeit not the one for which it was prepared. The other two names were *Trianthema glossostigmum* and *Wehlia staminosa*.

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[6] Inscribed 'To Sir Henry Barkly GCMG. K.CB., F.RS with regardful remembrance from Ferd. von Mueller' (National Library of Australia, PETHpam 533).

At the time the publication list was being revised, APNI entries for *Trianthema glossostigmum* and for *Dipteranthemum crosslandii* each had the comment:

> As stated by R.W.Home *et al.* (eds), *Regardfully Yours* 1: 637-638 (1998), Mueller's paper in the *Southern Science Record* volume 3 was not published in the journal. Mueller distributed the paper as page proofs and it is here considered effectively published under ICN Art. 29 (Melbourne Code, 2012)[7]

Based purely on the evidence in the 1998 edition of Mueller's publication list, these seemed suspect conclusions; they have now been deleted from APNI.[8]

The additional evidence provided in the entry in the present edition is more supportive of distribution of the text by Mueller. But distribution to collectors and correspondents is not sufficient evidence of effective publication: much depends on a careful analysis in terms of the criteria in the current *Code*: 'Publication is effected, under this Code, by distribution of printed matter (through sale, exchange, or gift) to the general public or at least to scientific institutions with generally accessible libraries' (Turland *et al.* 2018), Article 29). Any analysis to determine effective publication of *Diptheranthemum crosslandii* must consider whether part of the paper attached to an herbarium sheet, (i.e., not part of the Kew Library) when it is not known whether the whole extra-print was sent to Kew or only the page with the description, and a copy with unknown provenance now in the National Library of Australia, together constitute sufficient evidence of its having been placed in a qualifying location.

The other species described in the paper, *Wehlia staminosa*, was discussed by Craven (1987), p. 156:

> The original description of *Wehlia staminosa* was included in a paper due to be published in Volume 3 of *Southern Science Record*. Apparently the pages allocated to Mueller's paper were not included in the printing run and the paper was not actually published in the journal.[9] Mueller, however, received 'extra-prints' and these were distributed to some of his botanical correspondents. In this manner his new names have been published effectively under Article 29 of the International Code of Botanical Nomenclature ('extra-prints' are held in at least BRI, G and MEL, and probably are in K also). Mueller must have been satisfied with this unorthodox method of disseminating information as the paper does not seem to have been republished in a more formal fashion.

I have not found a copy of the whole item at Kew in Mueller's 'Opuscula' or attached to the sheets K821957 or PERTH01624644.[10] Unless the copies in Brisbane and Geneva have a clear provenance showing that Mueller sent copies of 84.13.18 to a 'generally accessible library', there is no evidence that supports Craven's judgement.

Secondly, item number 85.11.02:

> [Unpaginated extra-print] Definitions of some new Australian plants. *Southern Science Record*, new series, **1**. [This issue of the journal was never published. However, the republication in *Botanisches Centralblatt* in December, as '(From Wing's "Southern Science Record". Vol. 1 New Series 1885. Nov.)' indicates that Mueller sent a copy of the extra-print before the scheduled date of publication in Victoria. There is a copy of the sheet in the Australian National Library 'Botany leaflets', PETHpam 538.] [see also 85.12.05]

and its associated item 85.12.05:

> Definition of some new Australian plants. *Botanisches Centralblatt,* **24**(12-13): 373-374. [in Churchill *et al.* (1978) and Home *et al.* (1998) as 85.13.18] [see also 85.11.02]

In this case Mueller's descriptions were published, even though the intended publication was aborted. Nomenclaturists would need to consider whether a copy of the extra-print sent to the German journal, and the one in the National Library of Australia in a copy with unknown provenance and unknown date of distribution, is sufficient to satisfy the conditions for effective publication in the earlier print when considering the publication date of *Hibbertia holtzei*, *Tribulus forrestii* and *Babbagea scleroptera*. All three names were listed in APNI[11] as published in *Southern science record* and for the last two also in *Botanisches Centralblatt,* but without any warning about the fact that the issue of *Southern science record* listed was never published. The argument might also have to consider how much weight, if any, is to be given to Mueller himself later listing the *Southern science record* as the place of publication of these species (Mueller 1889, pp. 1, 22, 51 respectively, entry 89.12.03) (see below).

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[7] Here and below the entries quoted from the on-line APNI were accessed on 21 September 2022.

[8] All three names were listed in the hard-copy version of APNI (Chapman, 1991) as being validly published without any editorial commentary on the publication status of the paper in which they appeared. Anna Monro (CANB) suggested that the entries were probably made by analogy with the way *Wehlia staminosa* was treated after a subsequent re-examination of Craven (1987) (e-mail to Arthur Lucas, 30 October 2018).

[9] This is a misunderstanding of the publication history of the *Record*: the complete issue was missed, not just Mueller's paper, see Barnard (1899).

[10] A sheet from Herbarium Gardenerianum donated in 1970 to PERTH by the Benedictine Community of New Norcia.

[11] APNI entry under *Babbagia scleroptera*, the source documents give the name as *Babbagea scleroptera*.

Uncorrected proofs A third example, taking us back to the quotation at the head of this section, concerns a different issue, whether a proof known to have been amended before formal publication can be considered to be effectively published. Consider 83.04.07:

> [Proof sheets] Diagnoses of a new genus and species of Verbenaceae from Arnheim Land. [In his letter of 27 April 1883 to Ralph Tate, the editor of the journal, Mueller acknowledged receipt of the proofs, which Tate had sent to him before the paper was read on 1 May 1883. In his letter to Tate of 25 May 1883 Mueller requested an amendment, to change *Tatea acaulis* to *T. subacaulis*. That change, along with some other corrections in the description and the title, was made before publication in the journal. However, in his species listing in 84.03.06 and subsequently in 89.12.03, Mueller used his original name *Tatea acaulis*, as in the proof sheets, rather than the formally published *Tatea subacaulis*. There is a copy of the proof at Kew, accompanying K1067682. The specimen has one of Mueller's labels, with the name 'Tatea acaulis', annotated at Kew 'Rec^{d} 5/83', although it is not clear whether the proof sheet accompanied it. Another specimen, K1067681, with Tate's label using *T. subacaulis*, is annotated 'Com. R. Tate, 7/1884'. There is a copy of the proof in the Australian National Library 'Botany leaflets', PETHpam 538.] [see also 83.12.04]

and the associated 83.12.04:

> Diagnosis of a new genus of Verbenaceae from Arnhem's Land. *Transactions and Proceedings of the Royal Society of South Australia*, **6**: 33-34. [see discussion at 83.04.07]

There are two points of interest here. Firstly, which of the names has priority, the one in the proof sheets, or the one in the journal reflecting Mueller's considered(?) decision? It might be relevant that the copy at MEL of the proof sheet is a photocopy of the photocopy at AD supplied by K. In addition, Merrill (1951, p. 75) cited the name as *T. acaulis*, perhaps suggesting that there was a proof sheet at Harvard, but his 1950 note attached to K1067681 means that he saw that sheet, and thus the Kew copy of the proof. Munir (1984, p. 8), unknowingly but mistakenly described as a 'preprint' what Mueller referred to as 'the proof'. Once again, is there sufficient evidence here to consider the proof sheet effectively published, based on the proof attached to the herbarium sheet and a copy with unknown provenance and date of distribution in the Australian National Library? Secondly, if the proof sheet can be sustained as effectively published, is it appropriate to give priority to a name rejected by the author before it was formally published in the issue of the journal? Would that be compatible with the *Code?*

After considering these and similar examples the editors decided that it is beyond the scope of the bibliography to make decisions about whether items in it are effectively published and whether the taxon names given in them are validly published under the *Code*. That is a task for botanists, not historians. However, it is important to include these items in the new listing in some form as they would otherwise become 'fugitive publications'.

Proofs and extra-prints as effective publication

Once the decision was taken to include problematic proofs and extra-prints without attempting to determine whether they were effectively published, Mueller's practices ceased to be a bibliographic issue *per se*. However, preparing the third edition of Mueller's bibliography has inevitably meant considering the related literature. Thus, nomenclaturists considering the validity or priority of *Tatea acaulis / subacaulis* might consider the weight that can be placed on Mueller's use of the proof-sheet name in his second *Census* (Mueller 1889). The example suggests that Mueller listed the names when he had a commitment to publish but did not revise his index at final appearance of the journal. This is consistent with his treatment of the names mentioned in proofs or extra-prints that were not published in the intended source: three are outside the geographic range of the *Census;* one is a comment on an earlier name by Cunningham, one is not indexed, and the remainder are cited from non-existent *Southern science record* issues.[12] Names first printed in the gatherings of the planned but never completed second volume of *The plants indigenous to the Colony of Victoria* are treated in the same way. For example, in his *Census* the place of publication of *Acacia oswaldi* is 'F.M. Plants of Vict. ii, 24'. We know that sheets were sent successively to Bentham as they were printed,[13] and were quoted by him in Bentham (1863–1878), volume 2, which also cites the re-publication in Mueller's *Fragmenta*. For our example, *A. oswaldi*, in his *Census* Mueller also cited Bentham vol. 2, p. 384, and *Fragmenta*, volume 4, p. 5.[14]

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[12] Of these, Mueller did not publish elsewhere the names in 86.04.03 (*Euphorbia corynoclada, Melaleuca quadrifaria, Tristania longivalvis, Panax macdowalii*), but they are listed in *Just's Botanischer Jahresbericht* **14** Abt. 2, 218, (1886), as No. 608a, although the *Panax* is given as *P. madonelli.* Unusually, this *Panax* is not listed in either edition of his *Systematic census* … (83.03.04, 89.12.03).

On the face of it, Mueller, who was concerned, almost obsessed, with priority, intended to rely on these versions for the dates of his names. But that neglects his considered request to change the name of *Tatea acaulis / subacaulis*. Can a judgement of his intentions form part of a consideration of effective publication?

While not their main argument, Court, Cowan & Maslin (1994, p. 316) argue that although certainly distributed, the new names in the first 5 sheets of vol. 2 (in Mueller's bibliography as 63.13.06) of *The plants indigenous to the Colony of Victoria* were not effectively published under the 1988 *Code* as 'it appears to us unlikely that Mueller had any intention of making generally available to anyone the first few pages of an aborted publication, certainly not "to the general public" or "to botanists generally"'. Contrast this with the views of Craven (1987) quoted above: 'Mueller must have been satisfied with this unorthodox method of disseminating information as the paper does not seem to have been republished in a more formal fashion'. 'Intentions' are not part of the criteria in the current or earlier versions of the *Code* and it is difficult to see how they could be if the *Code* is to be clear.

Court, Cowan & Maslin implicitly acknowledge that the requirement for effective publication must depend upon the status of the recipient when the author distributed the text. Kew Gardens Library became state property and implicitly available to the public after William Hooker's collections comprising 'over 1 million herbarium specimens, 4000 volumes of publications, and about 29,000 letters dating from 1810 from over 4400 correspondents, bound in 76 volumes' were bought for the nation from William's heir Joseph Hooker in 1866 (FitzGerald 2004). It is certainly now accessible to both 'botanists generally' and 'the general public', but it would make nonsense of the *Code* if the five 63.13.06 sheets containing descriptions of *Acacia* species magically become effectively published as the status of the library changed. Similarly, effective publication of the material prepared for the *Southern Science Record* but never published there surely cannot have been effected when Petherick deposited his collection at the National Library of Australia from 1909. It is theoretically possible that he may have acquired some from a qualifying library, and secure provenance might show that a copy was originally deposited appropriately. However, provenance would be nigh impossible to establish for the National Library of Australia copies: the Library has no record of where Petherick originally acquired the items.[15]

Questions of effective publication must address several issues where evidence is difficult to obtain. The mere existence of copies outside MEL is not sufficient. I have not contacted all institutions that might hold copies of proofs or extra-prints to fully address the provenance questions in any specific case. However, the default condition surely must be that the effective publication is the journal where an item first appeared, whether the one originally intended or not, and that in cases where items exist only in the form of proofs or extra-prints, they must be considered 'not effectively published' unless very good provenance consistent with all the criteria of the *Code* is established. Hence the warning in the publication list:

> inclusion in Mueller's publication list ... does not in itself establish that the item has been 'effectively published'.

Fragmenta phytographiae australiae. Disconcertingly, individual fascicles of this major work could appear also to be problematic. In 1883, questions were raised about the distribution of Mueller's publications. In response to a question from James Service, the Premier of Victoria, the Government printer, John Ferres, reported that 500 copies of the *Fragmenta* were printed, and distributed 'To Departments, Visitors from other Countries, Horticultural Societies – on authorised requisitions, and by sale'. Ferres was reporting on the bound volumes, and Mueller provided additional information, including the comment:

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[13] Mueller to Bentham, 23 July 1863, RBG Kew, Kew correspondence, Australia, Mueller, 1858–70, ff. 110–111. Although copies have not been seen, Mueller almost certainly sent sheets to Sonder in Hamburg for distribution, see Mueller to Carl von Martius, 12 August 1863, Martuisiana, II, A, 2, Handschriften-Abteilung, Bayerische Staatsbibliothek, München.

[14] In addition to place and date of first publication of each species, the only literature Mueller cites in his *Census* is Bentham (1863–1878) and his own *Fragmenta*: he argued that by quoting the *Fragmenta* and *Flora Australiensis* 'all needful indications [of the literature] are afforded' (Mueller (1883, p. vi).

[15] 'Petherick, despite being an excellent cataloguer and bibliographer, did not keep detailed information on his own acquisitions or create a catalogue of his collection. They were most likely acquired by Petherick when he was based in London, probably prior to his bankruptcy in 1894, but could also have come into his collection after his return to Australia in 1908' (e-mail: Petherick Librarian (Andrew Sergeant) to R.W. Home, 13 February 2020). See also the entry for Edward Augustus Petherick in the *Australian Dictionary of Biography*, http://adb.anu.edu.au/biography/petherick-edward-augustus-4393, accessed 21 September 2022.

> of the loose numbers which constitute each volume of the fragmenta gradually, 25 copies are extraprinted, when ever each number appears, and given to me by Mr Ferres for use in my office and for sending to the principal amateurs at the time contributing unpaid to our collections. Each volume of the fragmenta requiring and having required from 2 to 4 years for its elaboration, with one or two exceptions.[16]

Two days later, Mueller corrected this statement:

> I have since learnt from my Assistant, that in later years we had received also 100 copies of the loose parts, altho' (so far as my memory carries me) we got only 25 copies of the separate unbound parts of the earlier volumes, the larger later requirements having arisen from the gradually augmented demands by botanic correspondents now nearly all over Australia and also widely abroad.[17]

Given the number of collectors acknowledged in a typical fascicle, it seems unlikely that 25 copies would allow libraries to have received copies of the fascicles as they were printed. So, especially for the early years, it could be that fascicles may not have been distributed in a manner which meets the criteria of the *Code*.

However, we know that, at least for volume 1, institutions received copies of individual fascicles as issued, see for example the description of a copy at the Linnean Society of London in Darragh & Lucas (2015, p. 303). We also know that the *Transactions and proceedings of the Royal Society of Victoria* records receipt of fascicles of the first seven volumes, and the first fascicle of the eighth (72.03.01), presented as they were published.[18] Fascicle 94 (82.12.03), the only one issued of volume 12 , was included in a list of donations during 1882 to the Library of the Royal Society of New South Wales 1883.[19] Furthermore, fascicles were being received at Kew. George Bentham received the text 'regularly transmitted to me as printed' (Bentham 1884, p. 305). Of course, although working at Kew, Bentham was a private individual, and it is not at all clear whether fascicles received by him after Kew library became a national establishment would have been made available to users of that Library. Bentham complained that 'the 4th and 5th vols of your Fragmenta have only come to us in the loose sheets sent to Sir W. Hooker as they came out and sometimes in proofs which appear to have been afterwards altered so that we have some difficulty in making them up complete.'[20] However, the language he uses suggests that Bentham did work collaboratively with the Hookers, and copies may have been available to those who wanted them. Similarly, in 1880 Mueller told Joseph Hooker that 'I send once more 89 & 91 of the frag.', that is, two years before the bound volume containing them was ready. The language again suggests that individual fascicles were routinely sent to Kew.[21]

Provided that libraries of societies, maintained for the benefit of members, and after 1865 the State-supported Kew library, qualify as 'generally accessible', there can be no doubt that the fascicles were effectively published as they were issued.[22]

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My co-editors of the revised publication list, and in their role as editors of *Correspondence of Ferdinand von Mueller*, have over many years provided direct or indirect assistance in considering the questions raised here. Rod Home checked files at MEL and read and commented on a penultimate draft. Anna Monro (CANB), who has responded to questions about how particular names have been treated in APNI, stimulated many of the

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[16] John Ferres to James Service, 8 May 1883; Mueller to James Service, 15 May 1883 (both P83/334, unit 4, VPRS 1163/P inward correspondence, VA 1123 Premier, Public Record Office, Victoria).

[17] Mueller to Ebenezer Thomas, 17 May 1883 (Unit 4, VPRS 1163/P1 inward correspondence, VA 1123 Premier, Public Record Office, Victoria).

[18] See, for example, the reports of the meeting of the Society on 21 August 1864 and 13 May 1872, *Transactions and proceedings of the Royal Society of Victoria*, respectively **6**, liv and **10**, 211.

[19] *Journal and proceedings of the Royal Society of New South Wales*, **16**, 264.

[20] George Bentham to Mueller, 19 April 1867 (RB MSS M4, Library, Royal Botanic Gardens Victoria)

[21] Mueller to Joseph Hooker, 10 August 1880 (RBG Kew, Kew correspondence, Australia, Mueller 1871-81, ff. 285-286).

[22] Although *Papuan plants* was issued in parts, as well as a bound edition for volume 1 (77.13.14), it is also unproblematic: parts are known to have been issued to public libraries (Report of the meeting of the Ballarat Public Library Committee, 6 June 1876, *Ballarat star*, 7 June 1876, p. 2).

thoughts here, and I have benefited from the careful reading by the *Muelleria* referees. As always, I am grateful to Paula Lucas for her critical reading of drafts. I of course am responsible for the interpretation of their comments.

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Muelleria

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ROYAL BOTANIC
GARDENS VICTORIA

Genome size estimates for Genomics for Australian Plants sequencing projects

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Abstract

Genome sizes were estimated with flow cytometry for candidate species for genome sequencing by the Genomics for Australian Plants consortium. Sampled species represent taxonomic groups where no or few genome sizes have previously been published. None of the three analysed Cunoniaceae produced clear peaks; it is possible that their genomes are very small. *Roepera similis* showed strong endopolyploidy, and because of the lack of a suitable standard and time constraints, no formal measurement was made. The first genome size estimates are presented for *Apodasmia brownii*, *Flindersia xanthoxyla*, *Gastrolobium racemosum*, *Hedycarya angustifolia*, *Hypolaena fastigiata*, *Pittosporum angustifolium*, *Phyllanthus gunnii*, *Quintinia fawkneri*, and *Ripogonum discolor*.

Keywords: endopolyploidy, flow cytometry, genome sequencing, genome size, polyploidy

Introduction

The Genomics for Australian Plants (GAP) consortium supports the sequencing and assembly of reference genomes for selected species as resources for the study of the evolution and conservation of the Australian flora (https://www.genomicsforaustralianplants.com/reference-genomes/). As of the time of writing in April 2023, 23 reference genome projects have commenced, with some already having proceeded to publication (Chen *et al.* 2022; McLay *et al.* 2022).

When considering the selection of species for sequencing, genome size is an important factor, because larger genomes require more investment in sequencing coverage and may be bioinformatically more challenging to assemble than species with small genomes (Hamilton & Robin Buell 2012). When choosing a species of a given genus for sequencing, a diploid with low DNA content per cell would therefore be preferred over a hexaploid relative, for example.

Currently, the most efficient approach for the determination of genome sizes is flow cytometry (Doležel *et al.* 2007). Living tissue, generally leaves, from a sample plant and a standard plant of known genome size, is finely chopped together in an extraction buffer to release intact cell nuclei. DNA in these nuclei is then stained with a fluorescent dye and observed in a flow cell (McKinnon 2018). The ratio of the fluorescence intensity peaks of the sample and of the standard allows the calculation of the absolute size of the sample genome in picograms.

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The laboratory of the Australian National Herbarium in Canberra has a flow cytometer that was set up to study ploidy levels in plants and has previously been used to estimate genome sizes, mostly for taxonomic or conservation genetic studies (Castelli *et al.* 2017; Chen *et al.* 2019).

In April 2023, the first author analysed a set of samples provided by Royal Botanic Gardens Victoria of candidate species for genome sequencing by the GAP consortium. The species were selected for flow cytometric analysis to represent so far under-studied groups with few or no published genome size measurements (Fig. 1). Here, the results of these analyses are reported because both the successful measurements and the taxonomically biased failures may be of interest to colleagues studying genome size evolution or planning future measurements in the same plant groups.

Methods

Leaf or (in the case of Restionaceae) stem samples of 13 species (Table 1) were collected at Royal Botanic Gardens Victoria's Melbourne and Cranbourne sites on 03 April 2023 and maintained in zip lock bags on ice. They were transported to Canberra on 04 April and analysed on 05, 06, and 12 April.

CyStain PI Absolute P (Sysmex Partex GmbH, Görlitz, Germany) was used for sample preparation following the manufacturer's instructions, except for halved reaction volumes. A tissue sample of approximately 0.5–1.0 cm^2 from both sample and standard was placed in 300 µL of extraction buffer in a Petri dish and chopped manually with a razor blade. The liquid was filtered through a 40 µm cell strainer and then transferred to a sample tube. The sample was mixed with 1,000 µL staining solution (staining buffer, propidium iodide, and RNAse). It was loaded into the flow cytometer (BD Accuri C6 Plus equipped with a 488 nm laser and a BD CSampler Plus, BD Biosciences, San Jose, CA, USA) and run at a flow rate of 14 mm/min per min. Histogram data were collected using the FL2 detector while eliminating events with a value of less than 80,000 on FL2-H. Analysis was performed with the BD Accuri C6 Software version 1.0.23.1. Soy (*Glycine max* (L.) Merr. 'Polanka', 2C = 2.50 pg) (Doležel *et al.* 1994) and pea (*Pisum sativum* L.

Table 1. Sample collection information, genome size standard used, and (where successful) genome size estimates for 13 Australian angiosperms. MEL accession numbers are those of voucher specimens deposited at the National Herbarium of Victoria, Melbourne. RBG accession numbers are those of the plants in the living collections of Royal Botanic Gardens Victoria (RBGM = Melbourne; RBGC = Cranbourne). *Hypolaena fastigiata* was a wild collection at RBGV Cranbourne.

Species	Family	Voucher	Standard	Measurements	Genome size (2C)
Callicoma serratifolia Andrews	Cunoniaceae	*D.J. Cantrill 2359* (MEL 2529498A; RBGM 501996)	NA	0	NA
Eucryphia lucida (Labill.) Baill.	Cunoniaceae	*G.D. Holmes 174* (MEL 2529901A; RBGC 194355)	NA	0	NA
Pullea stutzeri (F.Muell.) Gibbs	Cunoniaceae	*D.J. Cantrill 2364* (MEL 2529503A; RBGM 951570)	NA	0	NA
Gastrolobium racemosum (Turcz.) Crisp	Fabaceae	*G.D. Holmes 157* (MEL 2529504A; RBGC 114382)	pea	1	c. 1.69 pg
Hedycarya angustifolia A.Cunn.	Monimiaceae	*G.D. Holmes 175* (MEL 2529902A; RBGC 104027)	pea	3	1.38 (±0.02) pg
Quintinia fawkneri F.Muell.	Paracryphiaceae	*G.D. Holmes 155* (MEL 2529504A; RBGC 194330)	pea	3	2.35 (±0.06) pg
Phyllanthus gunnii Hook.f.	Phyllanthaceae	*D.J. Cantrill 2363* (MEL 2529502A; RBGM 170598)	pea	3	1.99 (±0.03) pg
Pittosporum angustifolium Lodd., G.Lodd. & W.Lodd.	Pittosporaceae	*G.D. Holmes 176* (MEL 2529903A; RBGC 014073.1)	soy	3	8.75 (±0.25) pg
Apodasmia brownii (Hook.f.) B.G.Briggs & L.A.S.Johnson	Restionaceae	*G.D. Holmes 177* (MEL 2529904A; RBGC 114125)	pea	3	1.55 (±0.04) pg
Hypolaena fastigiata R.Br.	Restionaceae	*G.D. Holmes 173* (MEL 2529905A)	2x pea, 1x soy	3	1.23 (±0.02) pg
Ripogonum discolor F.Muell.	Ripogonaceae	*D.J. Cantrill 2360* (MEL 2529499A; RBGM 941157)	pea	3	20.18 (±0.18) pg
Flindersia xanthoxyla (A.Cunn. ex Hook.) Domin	Rutaceae	*V. Stajsic 8999* (MEL 2457406A; RBGM 534828)	pea	3	0.86 (±0.03) pg
Roepera similis (H.Eichler) Beier & Thulin	Zygophyllaceae	*B.A. Swartz 15* (MEL 2478506A; RBGC 194032)	NA	0	c. 2.0–2.5 pg?

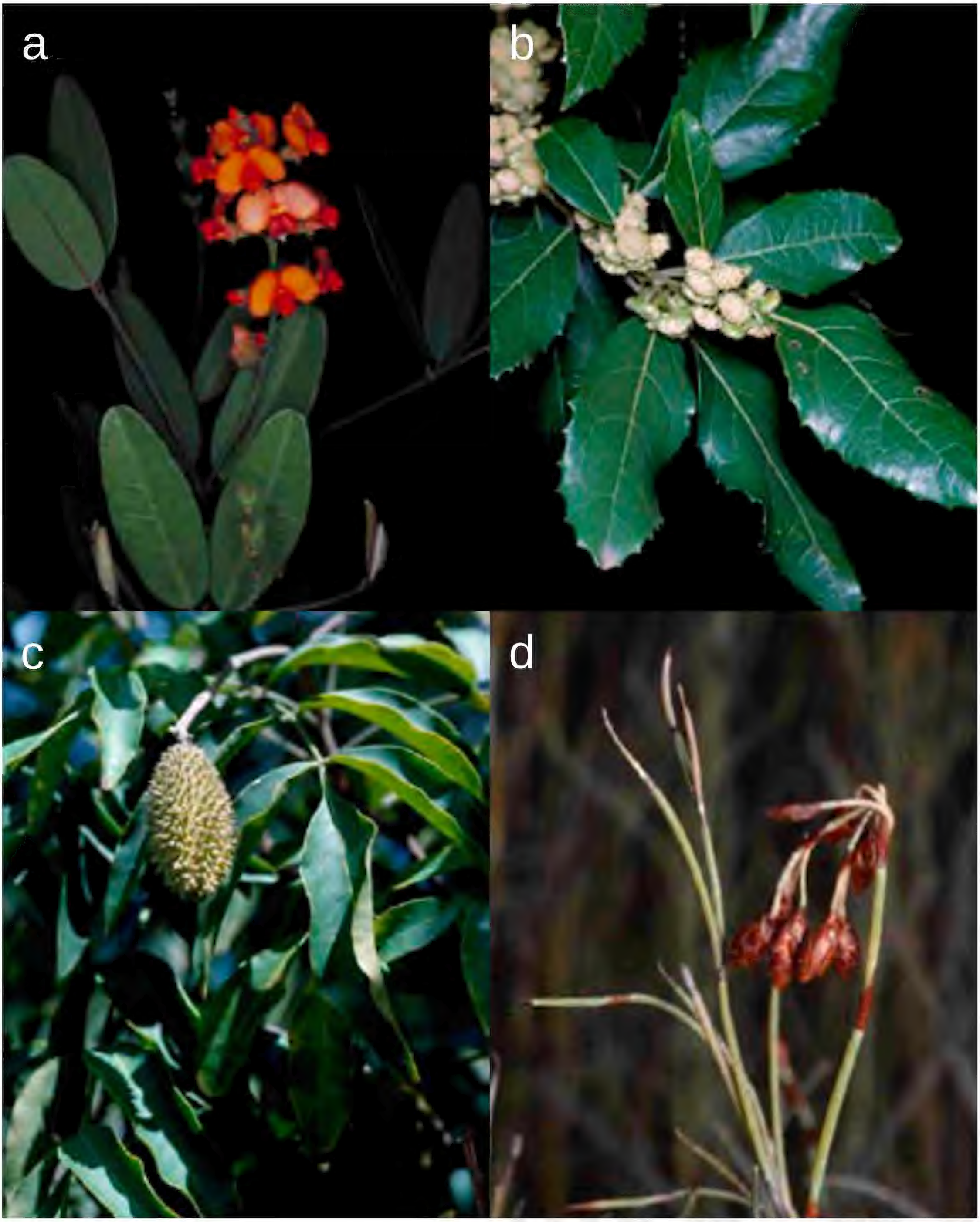

Figure 1. Four of the species whose genome sizes were estimated: a. *Gastrolobium racemosum* (Fabaceae). b. *Hedycarya angustifolia* (Monimiaceae). c. *Flindersia xanthoxyla* (Rutaceae). d. *Hypolaena fastigiata* (Restionaceae). Image sources: Australian Plant Image Index, photo numbers a.30121, a.27788, a.27376, and dig.35369, © Murray Fagg.

'UTAS Line 107', 2C = 9.09 pg) (Price 2010) were used as internal standards, depending on the size of the sample peak as determined by preliminary analyses without using standards.

Results and discussion

The estimated genome sizes, standards used, and sample voucher information are summarised in Table 1.

All three Cunoniaceae (*Callicoma serratifolia*, *Eucryphia lucida*, *Pullea stutzeri*) failed to produce distinct fluorescence peaks. It is possible that their genomes are very small, and peaks were obscured by the debris field (stained material other than intact nuclei; Fig. 2A). Unfortunately, the Plant DNA C-values database (https://cvalues.science.kew.org/search/angiosperm, accessed 07 April 2023) does not contain records for any other Cunoniaceae, so it remains unclear whether our interpretation is correct.

Roepera similis (Zygophyllaceae) showed strong endopolyploidy (Fig. 2B), *i.e.*, duplication of genomes without mitosis (Leitch & Dodsworth 2017). At the time of analysis, only two standards were at hand; the smaller one (soy) would have been too close to the *Roepera* 2C peak, and the larger one (pea) too close to the *Roepera* 8C peak. Therefore, no formal measurement was produced. The approximate position of the 2C peak just slightly left of where soy would have been suggests that the genome size of *Roepera similis* would be around 2C = c. 2.0–2.5 pg.

The analysis of *Gastrolobium racemosum* (Fabaceae) showed a large debris field that the 2C peak of the sample barely exceeded, leading to a poor measurement. Its genome size is estimated at 2C = c. 1.70 pg.

The remaining species were analysed using triplicate measurements, each time from a separate extraction: *Apodasmia brownii* (Restionaceae), 2C = 1.55 (±0.04) pg; *Flindersia xanthoxyla* (Rutaceae), 2C = 0.86 (±0.03) pg; *Hedycarya angustifolia* (Monimiaceae), 2C = 1.38 (±0.02) pg (Fig. 2C); *Hypolaena fastigiata* (Restionaceae), 2C = 1.23 (±0.02) pg; *Pittosporum angustifolium* (Pittosporaceae), 2C = 8.75 pg (±0.25) pg (Fig. 2D); *Phyllanthus gunnii* (Phyllanthaceae), 2C = 1.99 (±0.03) pg; *Quintiania fawkneri* (Paracryphiaceae), 2C = 2.35 (±0.06) pg; *Ripogonum discolor* (Ripogonaceae), 2C = 20.18 (±0.18) pg.

The genome size of *Pittosporum angustifolium* was unexpectedly large, as the only two measurements of the same genus in the C-values database were 2C = 0.92 pg and 2C = 1.22 pg, respectively (Hanson *et al.* 2001; Horjales *et al.* 2003). What is more, chromosome counts are available for numerous species of *Pittosporum* Banks ex Sol. (http://ccdb.tau.ac.il, accessed 12 April 2023), all consistently 2n = 24, including one of *P. angustifolium* (as *P. phillyreoides* DC.). Further research will be required to establish if this species is unexpectedly a polyploid complex or if genome size variation at the same ploidy level is strong in the genus.

A genome size of one picogram of DNA is equivalent to 0.978 gigabases (Doležel *et al.* 2003). However, for the purposes of sequencing and bioinformatic genome assembly, the relevant size is that of the haploid genome, so that the 2C values discussed here can be halved to obtain an approximate haploid size in gigabases.

Conversely, flow cytometry cannot provide information on ploidy level except through direct comparison of the genome sizes of close relatives, e.g., when finding that some samples have approximately twice or four times the genome size as others from the same genus. Because sampling was not designed in this way, the ploidy level of samples analysed in this study remains unknown.

Acknowledgements

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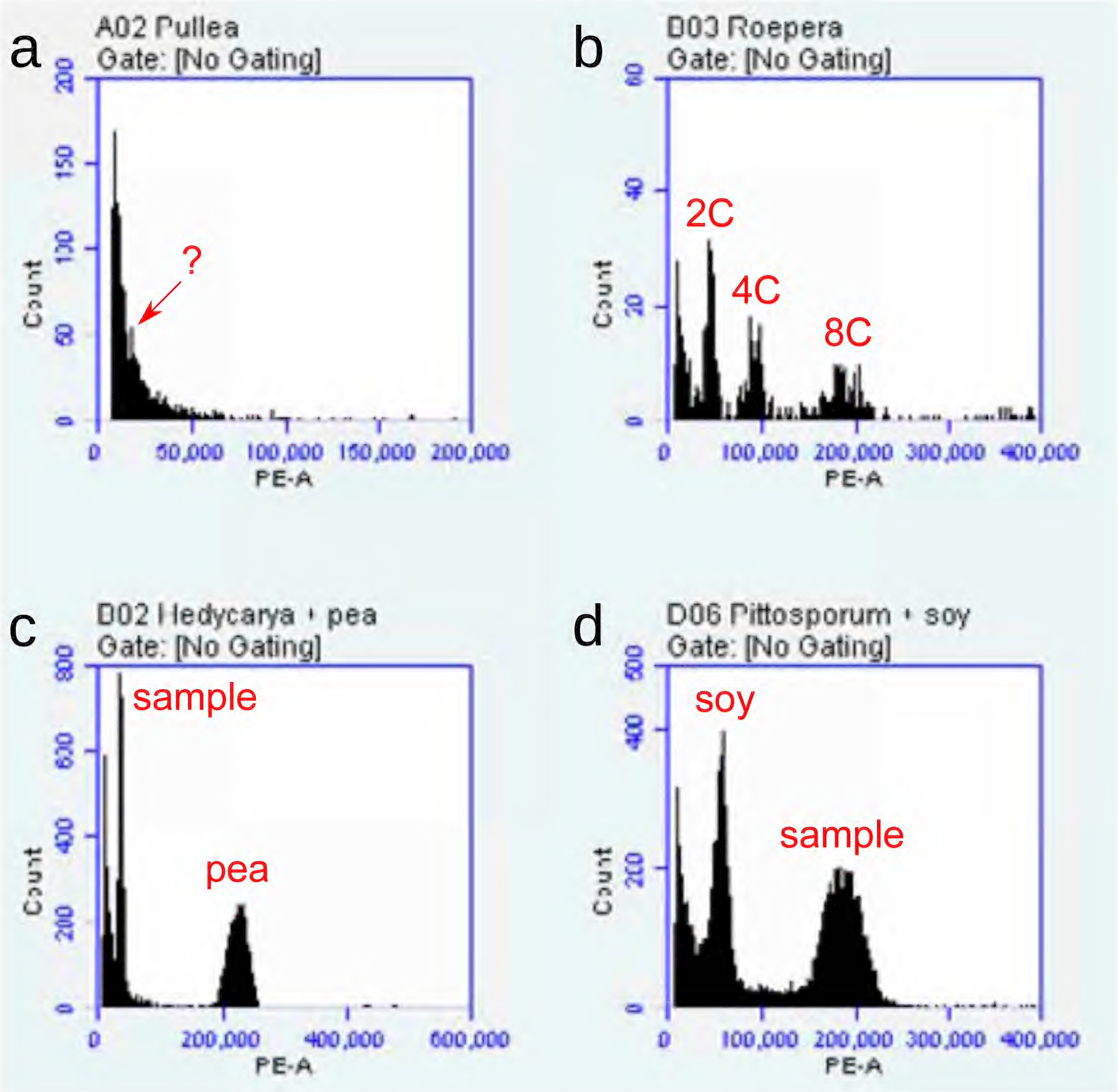

Figure 2. Flow cytometry histograms of selected analyses showing on the y-axis the frequency of nuclei with relative genome sizes indicated on the x-axis. a. *Pullea stutzeri* showing no peak that could with certainty be differentiated from the debris field on the left. The other two Cunoniaceae showed the same result. b. Endopolyploidy in *Roepera similis*, with strong 4C and 8C peaks. No standards were included in a. and b. c. *Hedycarya angustifolia* (2C = 1.38 pg) with pea (2C = 9.09 pg) as the standard. d. *Pittosporum angustifolium* (2C = 8.75 pg) showing an unexpectedly large with soy (2C = 2.5 pg) as the standard.

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A new classification of sections and series in *Eucalyptus* subg. *Eudesmia* (Myrtaceae)

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Abstract

The classification of sections and series in *Eucalyptus* subg. *Eudesmia* (R.Br.) L.A.S.Johnson & K.D.Hill is updated to align with results of a recent phylogenomic study. This revised classification involves recognition of one new section and two new series, namely *E.* sect. *Aurantistamineae* Bayly & R.Fowler, *E.* ser. *Phoeniceosae* (A.K.Gibbs & Ladiges) Bayly & R.Fowler, and *E.* ser. *Erythrocorythosae* (A.K.Gibbs & Ladiges) Bayly & R.Fowler.

***Keywords*:** taxonomy, phylogeny, eudesmid, new taxa

Introduction

Detailed infrageneric classifications of the eudesmid eucalypts, *Eucalyptus* subg. *Eudesmia* (R.Br.) L.A.S.Johnson & K.D.Hill, using the ranks of section, series and subseries, include those proposed informally by Hill & Johnson (1998) and formally by Blakely (1934), Chippendale (1988), Brooker (2000), Gibbs *et al.* (2009) and Nicolle (2022). Those classifications are based largely on morphology, but more recently have also been informed by molecular phylogenetic studies employing small numbers of molecular markers, e.g., variously those of Steane *et al.* (2002, 2011), Gibbs *et al.* (2009) and Thornhill *et al.* (2019).

Here we present a new classification of sections and series in *E.* subg. *Eudesmia* based on recent phylogenomic analyses of McLay *et al.* (2023). That study, based on a dataset of 73 eudesmid samples and sequences of 522 nuclear genes, and a smaller set of plastid genes, provided new insight into eudesmid relationships and resolved some infrageneric taxa, recognised in the recent classifications of Gibbs *et al.* (2009) and Nicolle (2022), as polyphyletic. In particular, in the nuclear phylogeny, *E.* sect. *Complanatae* Brooker, sensu Nicolle (2022) or Gibbs *et al.* (2009), was resolved as polyphyletic because of the placement of *E. tetrodonta*

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F.Muell. with members of *E.* sect. *Limbatae* Brooker, and *E.* ser. *Heteropterae* Maiden was polyphyletic because *E. erythrocorys* F.Muell was placed outside that group. In addition, placement of different accessions of *E. gittinsii* Brooker & Blaxell in multiple positions in the nuclear tree rendered subser. *Tetraedrae* Brooker, sensu Gibbs *et al.* (2009), polyphyletic.

Our new classification for the eudesmids is intentionally phylogenetic, recognising only monophyletic groups, and focusing only on clades resolved in the nuclear phylogeny with robust support; we have not focussed on analyses of plastid DNA, which are frequently discordant with nuclear phylogenies and with classification of eucalypts (McKinnon *et al.* 1999; Bayly 2016; Schuster et al. 2018; Fahey *et al.* 2021), including in *E.* subg. *Eudesmia* (McLay *et al.* 2023). The nuclear DNA analyses of McLay *et al.* (2023) identified three main eudesmid clades (labelled by McLay *et al.* as Clades A, B and C), which are each recognised here at the rank of section (Figure 1). We recognise robust clades within those groups at the rank of series, largely using existing series names, but establishing new series names for two groups previously recognised at the rank of subseries. We do not use the rank of subseries, primarily because most series are small, and we don't see great value in recognising subseries with small numbers of species. Additionally, there is taxonomic and phylogenetic uncertainty (discussed below) in the largest series, *E.* ser. *Heteropterae*, and the presence of potentially polyphyletic species hinders the recognition of subseries in that group.

Taxonomy

We recognise the following sections and series in *Eucalyptus* subg. *Eudesmia*. The relationship of these taxa to the eudesmid phylogeny of McLay *et al.* (2023) is shown in Figure 1.

Eucalyptus sect. *Aurantistamineae* Bayly & R.Fowler, sect. nov.

Type: *Eucalyptus miniata* Schauer

Diagnostic description: Distinguished from other sections of *Eucalyptus* by the combination of: orange staminal filaments; calyx fused to the corolla and evident, in bud, as four small teeth at apex of corolline operculum.

Etymology: The name refers to the conspicuous orange stamens that characterise this group within *E.* subg. *Eudesmia*.

Eucalyptus ser. *Phoeniceosae* (A.K.Gibbs & Ladiges) Bayly & R.Fowler, stat. nov.

Basionym: *Eucalyptus* subser. *Phoeniceosae* A.K.Gibbs & Ladiges, *Austral. Syst. Bot.* 22(3):174 (2009).

Type: *E. phoenicea* F.Muell.

Included species: *E. ceracea* Brooker & Done, *E. phoenicea*

Notes: Nicolle (2022) recognises Queensland populations of *E. phoenicea* as a distinct, undescribed species, "*E.* sp. Battlecamp (*Nicolle 1789*)" that would also be included in this series.

Eucalyptus ser. *Miniatae* Blakely

Type: *Eucalyptus miniata* Schauer

Included species: *E. chartaboma* D.Nicolle, *E. miniata*

Notes: Also included in this series is *E. aurantiaca* F.Muell., which is generally regarded as a synonym of *E. miniata* (e.g., Chippendale 1988; Hill & Johnson 1998; Slee *et al.* 2006, 2022; CHAH 2023), but is treated as a distinct species by Nicolle (2022). We have not assessed the distinctiveness of these taxa, but samples used in the nuclear DNA phylogeny (Figure 1) likely fit with Dean Nicolle's concept of *E. aurantiaca* (based on their field location), rather than *E. miniata sensu stricto* (D. Nicolle pers. comm., 2022).

Eucalyptus sect. *Reticulatae* Brooker

Type: *Eucalyptus baileyana* F.Muell.

Eucalyptus ser. *Scutelliformes* Maiden

Lectotype and only included species: (designated by Chippendale 1988, p. 505) *E. baileyana* F.Muell

Eucalyptus ser. *Similes* A.K.Gibbs & Ladiges

Type: *E. similis* Maiden

Included species: *E. lirata* W.Fitzg. ex Maiden, *E. similis*

Eucalyptus sect. *Limbatae* Brooker

Type: *Eudesmia tetragona* R.Br. [considered an intergrade between *E. extrica* D.Nicolle and *E. pleurocarpa* Schauer; Nicolle (2000)]

Notes: The section names *Limbatae* Brooker and *Complanatae* Brooker could both apply to this group and have equal priority, being simultaneously published in the same work. Here we opt to use the name sect. *Limbatae*, thus establishing priority under Art. 11.5 of the International Code of Nomenclature for Algae, Fungi and Plants (Turland *et al*. 2017), because that usage more closely matches circumscriptions used in recent classifications.

Eucalyptus ser. *Tetrodontae* Chippend.

Type**:** *Eucalyptus tetrodonta* F.Muell.

Included species**:** *E. tetrodonta*, *E. megasepala* A.R.Bean

Eucalyptus ser. *Edentatae* Brooker

Type and only included species**:** *Eucalyptus gongylocarpa* Blakely

Eucalyptus ser. *Jucundae* Chippend.

Type*:* *Eucalyptus jucunda* C.Gardner

Included species**:** *E. jucunda*, *E. roycei* S.G.M.Carr, D.J.Carr & A.S.George

Eucalyptus ser. *Erythrocorythosae* (A.K.Gibbs & Ladiges) Bayly & R.Fowler, stat. nov.

Basionym**:** *Eucalyptus* subser. *Erythrocorythosae* A.K.Gibbs & Ladiges, *Austral. Syst. Bot.* 22(3):175 (2009).

Type and only included species**:** *Eucalyptus erythrocorys* F.Muell.

Eucalyptus ser. *Ebbanoenses* Chippend.

Type and only included species**:** *E. ebbanoensis* Maiden

Eucalyptus ser. *Odontocarpae* Chippend.

Type**:** *E. odontocarpa* F.Muell.

Included species**:** *E. odontocarpa*, *E. gamophylla* F.Muell.

Eucalyptus ser. *Heteropterae* Maiden

Lectotype**:** (designated by Chippendale 1988, p. 498) *Eucalyptus tetragona* (R.Br.) F.Muell. [considered an intergrade between *E. extrica* D.Nicolle and *E. pleurocarpa* Schauer; Nicolle (2000)]

Included species**:** *E. conveniens* L.A.S.Johnson & K.D.Hill, *E. eudesmioides* F.Muell., *E. extrica*, *E. gittinsii* Brooker & Blaxell, *E. pallida* L.A.S.Johnson & K.D.Hill*, E. pleurocarpa, E. selachiana* L.A.S.Johnson & K.D.Hill

Notes**:** This is the largest series recognised in this classification. The included species were separated by Gibbs *et al*. (2009) into three subseries (*Tetraedrae* Brooker, *Pleurocarpae* A.K.Gibbs & Ladiges, and *Convenienses* A.K.Gibbs & Ladiges) of a more broadly defined sect. *Heteropterae*, but were included as the only members of a single subseries (*Tetraedrae*) by Nicolle (2022). They are not divided into subseries here, because of uncertainty about species boundaries and because of low support for some relationships in the group.

Species taxonomy in this group requires attention, and our list of included species is intended to be indicative, rather than making firm assertions about the species that should be recognised. Here, as done by McLay *et al*. (2023), we follow the taxonomy of the Australian Plant Census (CHAH 2023) and Euclid (Slee *et al*. 2006), in including both *Eucalyptus selachiana* L.A.S.Johnson & K.D.Hill and *E. pallida* L.A.S.Johnson & K.D.Hill, informally, as subspecies under *E. eudesmioides*. An alternative classification is proposed by Slee *et al*. (2020) who recognise *E. selachiana* as distinct, but continue to include *E. pallida* under *E. eudesmioides*. Nicolle (2000) also indicated that *E. pallida* should be included under *E. eudesmioides*, but the list of Nicolle (2022) includes all three species. The nuclear phylogeny (Figure 1) does not resolve these taxa as distinct from each other (or from *E. gittinsii* subsp. *gittinsii*), but support in that part of the tree is low, and we have not investigated the morphology of these taxa in sufficient detail to make firm recommendations on species limits.

Previous discussions of other species in *E.* ser. *Heteropterae* suggest that introgression is widespread and contributes to difficulties in species delimitation. *Eucalyptus conveniens* is considered, on morphological grounds, to be a stable intergrade between *E. gittinsii* (subsp. *illucida*) and *E. pleurocarpa* (Hill & Johnson 1998; Nicolle 2000, 2022; French & Nicolle 2019), which could be consistent with the positions of those taxa in the nuclear phylogeny. Intergrades are also reported between *E. extrica* and *E. pleurocarpa*, and have morphology matching the type of *E. tetragona*, as indicated by Brooker (2000) and Nicolle (2000). Intergrades between *E. gittinsii* subsp. *illucida* and *E. eudesmioides* were noted by Nicolle (2000); Nicolle & French (2019) also indicated that *E. gittinsii* subsp. *gittinsii* and *E. eudesmioides* sometimes interbreed, forming populations of plants with intermediate characteristics. The close association of the last two taxa in the nuclear phylogeny (Figure 1), as well as in the plastid phylogeny of McLay et al. (2023), hints at the possibility of a close relationship, if not gene flow between them, but relationships in that part of the tree are not well supported.

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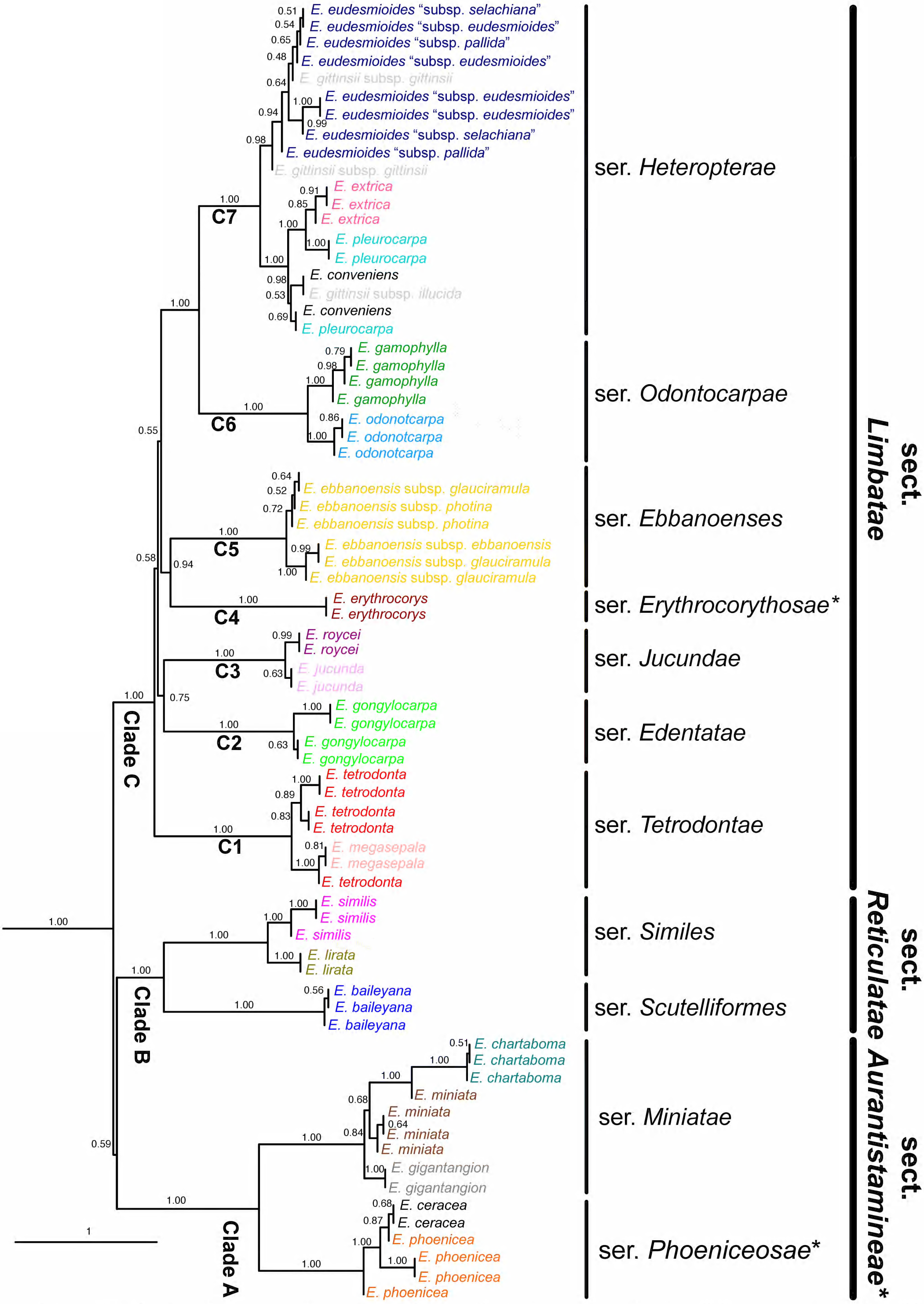
0.51 E. eudesmioides "subsp. selachiana"
0.54 E. eudesmioides "subsp. eudesmioides"
0.65 E. eudesmioides "subsp. pallida"
0.48 E. eudesmioides "subsp. eudesmioides"
0.64 E. gittinsii subsp. gittinsii
1.00 E. eudesmioides "subsp. eudesmioides"
0.94
0.99 E. eudesmioides "subsp. eudesmioides"
E. eudesmioides "subsp. selachiana"
0.98 E. eudesmioides "subsp. pallida"
E. gittinsii subsp. gittinsii
0.91 E. extrica
0.85 E. extrica
1.00 E. extrica
1.00 E. pleurocarpa
E. pleurocarpa
1.00
0.98 E. conveniens
0.53 E. gittinsii subsp. illucida
0.69 E. conveniens
E. pleurocarpa
1.00 C7
1.00
ser. Heteropterae
0.79 E. gamophylla
0.98 E. gamophylla
1.00 E. gamophylla
E. gamophylla
0.86 E. odonotcarpa
1.00 E. odonotcarpa
E. odonotcarpa
1.00 C6
0.55
ser. Odontocarpae
0.64
0.52 E. ebbanoensis subsp. glauciramula
0.72 E. ebbanoensis subsp. photina
E. ebbanoensis subsp. photina
0.99 E. ebbanoensis subsp. ebbanoensis
E. ebbanoensis subsp. glauciramula
1.00 E. ebbanoensis subsp. glauciramula
1.00 C5
0.58
0.94
ser. Ebbanoenses
1.00 E. erythrocorys
E. erythrocorys
C4
ser. Erythrocorythosae*
0.99 E. roycei
E. roycei
0.63 E. jucunda
E. jucunda
1.00 C3
ser. Jucundae
0.75
1.00 E. gongylocarpa
E. gongylocarpa
0.63 E. gongylocarpa
E. gongylocarpa
1.00 C2
ser. Edentatae
1.00 E. tetrodonta
0.89 E. tetrodonta
0.83 E. tetrodonta
E. tetrodonta
0.81 E. megasepala
1.00 E. megasepala
E. tetrodonta
1.00 C1
ser. Tetrodontae
1.00 Clade C
sect. Limbatae
1.00 E. similis
1.00 E. similis
E. similis
1.00
1.00 E. lirata
E. lirata
ser. Similes
0.56 E. baileyana
E. baileyana
E. baileyana
1.00
ser. Scutelliformes
1.00 Clade B
sect. Reticulatae
1.00
0.51 E. chartaboma
1.00 E. chartaboma
E. chartaboma
1.00 E. miniata
0.68 E. miniata
0.64
0.84 E. miniata
E. miniata
1.00 E. gigantangion
E. gigantangion
1.00
ser. Miniatae
0.68 E. ceracea
0.87 E. ceracea
1.00 E. phoenicea
1.00 E. phoenicea
E. phoenicea
1.00 E. phoenicea
ser. Phoeniceosae*
0.59
1.00
Clade A
sect. Aurantistamineae*
1

Figure 1. New classification of sections and series in *Eucalyptus* subg. *Eudesmia* aligned with a phylogenetic tree produced by McLay *et al*. (2023). Newly named taxa are indicated by an asterisk. The tree is an ASTRAL phylogeny based on 522 nuclear gene trees. Values on branches represent local posterior probability (LPP); major clades (A, B and C1–C7) identified and discussed by McLay *et al*. (2023) are also labelled. Species labels are colour-coded; see McLay *et al*. (2023) for collecting details of each accession (the order of samples in this tree is identical to that in Fig. 2 of McLay *et al*. 2023).

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